Carina Alexandra Félix Figueiredo

Licenciatura em Bioquímica

Development of biological and synthetic affinity ligands for Human Serum Albumin

Dissertação para obtenção do Grau de Mestre em Bioquímica

Orientador: Professora Doutora Ana Cecília Afonso Roque

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FACULDADE DE CIÊNCIAS E TECNOLOGIA
UNIVERSIDADE NOVA DE LISBOA

Setembro 2016

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Abstract

This work explored the development of synthetic and biological affinity ligands for the capture and purification of Human Serum Albumin (HSA).

Three synthetic affinity ligands based on the Ugi and Triazine reaction (A3A2, A6A5 and C7A3), previously selected as lead ligands for binding HSA, were tested towards the optimization of the binding and elution conditions to capture and recover purified samples of HSA and HSA from human plasma. It was found that the final purity of recovered HSA purified by chromatographic resins modified with ligands A3A2 and A6A5 was 37 and 93%, respectively. The static binding affinity constant of A6A5 adsorbent for HSA was determined, being in the 10^{6}-10^{5} M^{-1} range as expected for affinity purification systems.

Three proteins based on engineered WW domains, previously evolved to bind to HSA and IgG, were purified and re-assessed for target binding by ELISA assays. The proteins were available as GFP-fusion proteins and were firstly purified by affinity chromatography. After purification, two ELISA assays were conducted to assess the selective binding between the engineered WW domains and the targets, yielding no measurable binding between the species.

Keywords: Human Serum Albumin, biological and synthetic affinity ligands, GFP fusion proteins, ELISA

Resumo

Este trabalho explora o desenvolvimento de ligandos biológicos e sintéticos de afinidade para captura e purificação da Albumina do Soro Humano (ASH).

Três ligandos de afinidade sintéticos baseados nas reações de Ugi e Triazine (A3A2, A6A5 e C7A3), anteriormente selecionados como os principais ligandos para ligação com ASH, foram testados através da otimização das condições de binding e eluição para capturar e recolher amostra de HSA purificada e ASH do plama humano. Foi encontrado a pureza final da recolha da HSA pelas resinas de cromatografia modificadas com os ligandos A3A2 e A6A5 sendo de 37 e 93%, respectivamente. A constante estática de afinidade de ligação do absorvente A6A5 para a ASH foi determinada, encontrandeo-se no intervalo 10^6-10^5 M^{-1} como e espectável para sistemas de purificação por afinidade.

Três proteínas baseadas na engenharia nos domínios dos WW, anteriormente desenvolvidos para ligar a ASH e IgG, foram purificadas e reavaliados por ligação ao alvo por ensaios de ELISA. As proteínas foram desponibilizadas como proteínas com fusão a GFP e foram primeiramente purificadas por cromatografia de afinidade. Posteriormente à purificação, dois ensaios de ELISA foram conduzidas para avaliar a ligação selectiva entre os domínios WW e os alvo, produzindo nenhuma ligação mesurável entre as espécies.

Palavras-chave: Albumina do Soro Humano, ligandos de afinidade biológicos e sintéticos, GFP proteínas de fusão, ELISA

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List of abbreviations

Aas- Amino acids residues

ABDs- Albumin Binding Domains

APS – ammmonia persulfate

BCA – bicinchoninic acid assay

BSA – bovine serum albumin

DMF- Dimethylformamide

E.coli – *Escherichia coli*

Eq- Equivalent

FcRn- Neonatal Fc receptor

GFP- Green Fluorescent Protein

HCl- Hydrochloric acid

HSA- Human Serum Albumin

IgG- Immunoglobulin G

IPTG – isopropyl β-D-1-thiogalactopyranoside

Ka – affinity constant

LB – Luria Broth

N/A- Not Available

NaCl- Sodium chloride

NaOH- Sodium hydroxide

OD600nm – optical density measured at 600nm

pAP006 – expression vector containing the GFP gene

PBS – buffer containing 10mM sodium phosphate, 150mM NaCl, pH7.4

PDB – RCSB Protein Data Bank

pDNA – plasmid DNA

PEG – poly(ethylene glycol)

RT – room temperature

SDS-PAGE - Sodium dodecyl sulfate polyacrylamide gel electrophoresis

TAE – buffer containing 40mM Tris-base, 20mM glacial acetic acid, 1mM EDTA, pH8.4

TEMED – tetramethylethylenediamine

WW domain- Tryptophan tryptophan domain

Chapter 1.
LITERATURE REVIEW

This first chapter describes the unique properties, in terms of structure and function, and the several biotechnological and biomedical applications for Human Serum Albumin (HSA). There is a particular focus on natural and synthetic HSA binders and its applications.

I.1 - Historical Perspective

The name albumin evolved from the more general term, *albumen*, the early German word for protein. Its derived was Latin, *albus,* which means white, the color of that part of an egg surrounding the yolk when it is cooked [1].

Some pathophysiological properties of human serum albumin (HSA) was documented in 1822 by Hippocrates of Cos, a foamy urine, possibly caused by the presence of HSA, which indicates kidney disease [1]. Over the years the importance of HSA has increased as well as studies related to its application to the health level. In 1932, HSA was separated from plasma proteins [2] and in 1934, HSA was crystallized pass, after 1940, HSA was purified for intravenous use as a blood substitute [3];

Only in 1960, the HSA ‘‘domain’’ structure was proposed [4] . The primary structure of HSA was deduced [5] , and the characterization of specific drug binding sites started [6], in 1975. Afterward, in 1979, the HSA gene was isolated and in 1981, the nucleotide sequence of HSA cDNA was reported [7] . With the help of this studies, in 1986, the complete gene sequence of HSA was determined [8].

The ‘‘heart-shaped’’ three-dimensional structure of HSA was determined in 1992 [9], and in 1999, human serum heme-albumin (HSA–heme) was engineered to become a O2 carrier [10]; and starting from 2001, the detoxifying role of HSA–heme–Fe towards reactive nitrogen species (RNS) and reactive oxygen species (ROS) was underlined [1],[11].

I.2 - Human Serum Albumin (HSA)

Human serum albumin (HSA) is the most abundant protein in the circulatory system. Because of its availability, low cost, stability, and unusual ligand binding properties, serum albumin has been one of the most extensively studied and applied proteins in biochemistry [12].

Serum albumin is a member of a family of homologous proteins characterized by structural features which includes namely α-fetoprotein (AFP), afamin (AFM – also named α-albumin) and vitamin D binding protein (DBP)[13].

I.2.1 - Albumin production, distribution and metabolism

Albumin corresponds to 60% of the total protein present in the plasma of normal healthy individuals, is the main determinant of plasma oncotic pressure, and plays an important role in modulating the distribution of fluids between body compartments [1]. It is synthesized predominantly in the liver, and rapidly secreted from the hepatocytes resulting in approximately 13–14 g of albumin entering the intravascular space every day [14].

HSA is synthesized only in a suitable nutritional, hormonal, and osmotic environment. The colloid osmotic pressure and the osmolality of the interstitial liquid around the hepatocytes is the most important regulator of HSA biosynthesis. For example, in the case of the acute-phase reaction, the response of the body to a stressful trauma, burn or acute infection, the concentration of the hepatic HSA mRNA is depressed [1]. This reduction in mRNA concentration, caused by the decrease in gene transcription, is seen in the acute-phase reaction and mediated by cytokines, mainly interleukin-6 (IL-6) and tumor necrosis factor α (TNF-α) [15].

The normal human plasma albumin concentration is 34–54 g/L and with an average plasma volume of 2.5–3.0 L for a 70 kg person, the average intravascular albumin mass is 113–126 g. HSA circulates from the blood across the capillary wall into the interstitial compartments, including cerebrospinal fluid, and returns to the blood through the lymphatic system with a circulation half-life of approximately 16 hours. The rate of HSA degradation depends on its concentration, for example protein and calories deprivation accelerates HSA catabolism [1].

I.2.2 - Structure features

HSA is synthesized in the hepatocyte with the production of pre-pro-albumin that is modified into pro-albumin in the lumen of the endoplasmic reticulum [1].

The mature plasma protein, is constituted by a single chain of 585 residues, with a molecular weight of 66,438 Da [16]. Through its sequence, it is possible to see that HSA contains a single Trp residue at position 214, and Cys and Lys residues are abundant. These 35 Cys residues that HSA contain form 17 disulfide bridges, except the Cys residue located at position 34. The disulfide bridges contribute to the stability of HSA and make its long biological life-time. In HSA, the acidic amino acid residues outnumber the basic ones in HSA, resulting in a negative net charge per molecules of about -15 at pH 7 [14].

The secondary structure of HSA is dominated by α-helices, about 68 %, without any β-sheet conformation. Serum albumin is arranged in a globular heart shaped conformation (Fig 1.B) containing three homologous domains indicated as I (1-195), II (196-383), and III (384-585) [17]. Each domain includes ten helices that are packed in two separate subdomains (named A and B) constituted of six (h1–h6) and four (h7–h10) α-helices, respectively, connected by a long extended loop [18].

Due to the high number of acidic and basic residues, HSA suffers reversible conformational transitions at different pH values. At pH lower than 2.7, HSA occurs in the extended conformation. Between pH 2.7 and 4.3, HSA assumes the fast-migrating form, characterized by an increase in viscosity, lower solubility, and the loss of α- helices. Between pH 4.3 and 8, HSA features the N form that is characterized by the heart-shaped structure. At pH greater than 8, HSA changes its conformation to the basic form characterized by the loss of α-helix [1].

I.2.2.1 - Drug Binding Sites of HSA

HSA binds a number of relatively insoluble endogenous compounds such as unesterified fatty acids, bilirubin, and bile acids and thus facilitates their transport throughout the circulation. HSA is also capable of binding a wide variety of drugs [16].

Typically, drugs bind to one or a very few high-affinity sites with association constants in the range of 10^4-10^6 M^{-1}. In the albumin, most drugs bind with a high affinity to one of two sites, referred to as site I and site II (Fig 1 C). Site I ligands appear to be dicarboxylic acids and/or bulky heterocyclic molecules with a negative charge localized in the middle of the molecule [16].

. Ligands as large as bilirubin can be bound, and the independent binding of two different compounds to the site can occur. The fact that ligands with very different chemical structures bind to the region with high affinity indicates that the site is adaptable [16]. Site I is formed as a pocket in subdomain IIA, the inside wall of the pocket is formed by hydrophobic side chains, however the entrance to the pocket is surrounded by positively charged residues [16].

Ligands that bind to site II, called the indolebenzodiazepine site are aromatic carboxylic acids with negatively charges acidic groups. Site II seems to be smaller or more constricted than drug site I because no large ligands apparently bind to it. Site II is a pocket that is formed in subdomain IIIA, and it appears to be less flexible because binding by stereoselectivity [16].

I.2.2.2 - Ligand binding properties

HSA is able to bind up to seven equivalents of long chain fatty acids (FAs; which represent the primary physiological ligands) at multiple binding sites with different affinity.

The various FAs are localized in different regions of albumin and have different characteristics. The FA1 is a cavity within subdomain IA. FA2 is situated at the interface between subdomains IA, IB and IIA. The FAs, FA3 and FA4 contribute to the formation of a large cavity in subdomain IIIA. FA5 is located within subdomain IIIB with the polar head orientated towards subdomain IIIA. Whereas, FA6 is a solvent-accessible linear slot situated at the interface between subdomain IIA and IIB. Lastly, FA7 is a large flattened cavity comprised within subdomain IIA. FAs that are accommodated inside FA6 and FA7 are a consequence of hydrophobic interactions. FA binding sites also provide accommodation for several endogenous and exogenous ligands. (Table 1) [1].

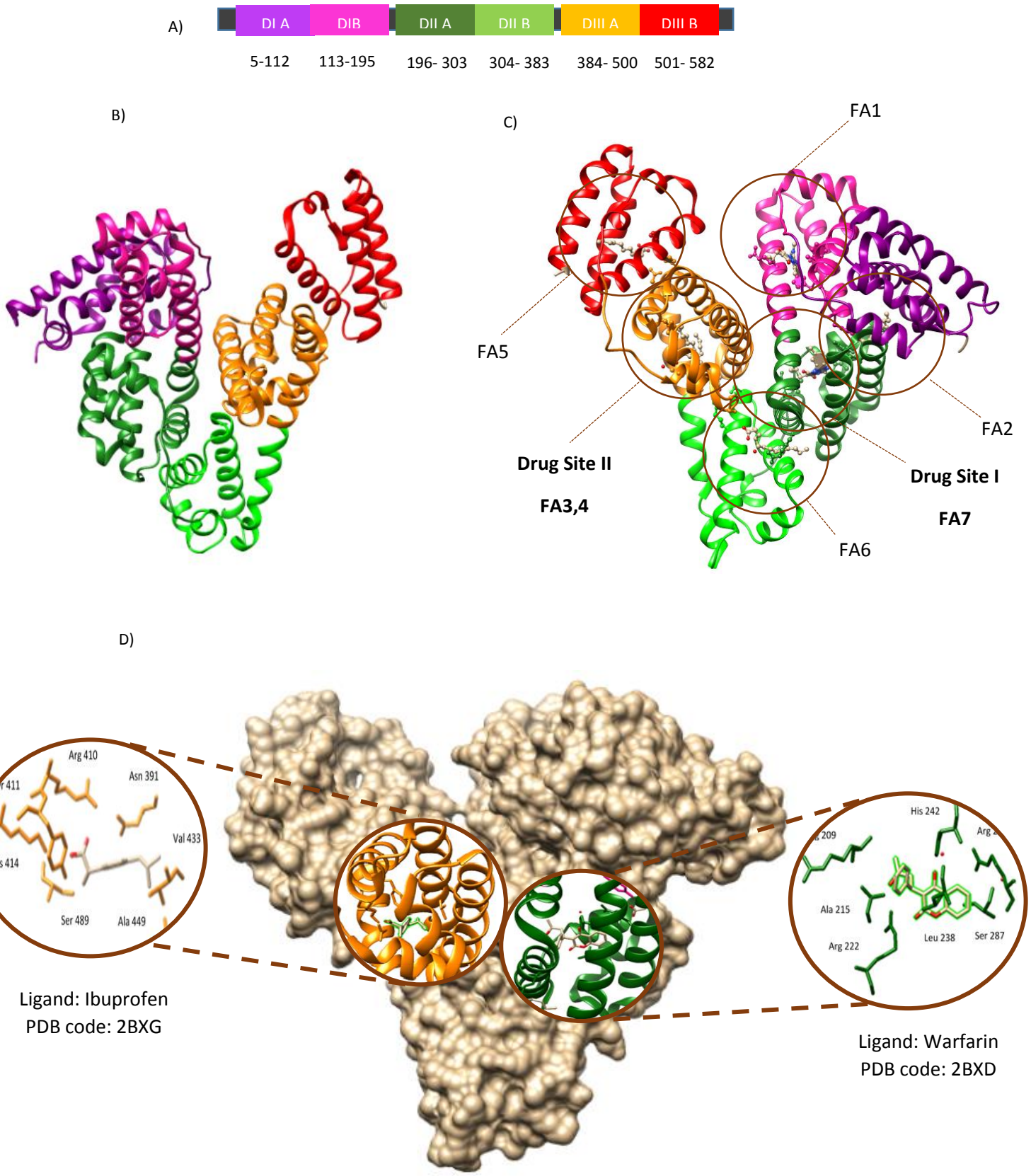

Figure 1-Structure of HSA with ligand binding sites. Representation in ribbon models of HSA. B) Determined in 1999 by x-ray crystallography with a high resolution 2.5 Å. Structure with the subdomains in different colors ((A) sequence of subdomains with respective color) PDB code 1AO6.**C)** Determined in 2005 by X-ray crystallography with a high resolution (2.5 Å). D) Structure with anticoagulant (warfarin PDB code 2BXD) and anti-inflammatory (Ibuprofen PDB code 2BXG). The six subdomains of HSA are colored differently (A)). Image built in UCSF Chimera software (PDB code: 2BXI)

Table 1 - List of endogenous and exogenous molecules that bind to HSA.

Endogenous ligands						
Ligands	Examples	Ligand binding sites	PDB code	Resolution (Å)	Ka (M^{-1})[e]	References
Fatty acids	Myristic acid	FA1, FA2, FA3, FA4, FA5,	1BJ5	2.5	5.31×10^{7}	[19]
Hormones	Thyroxine	Tr-1[a], Tr-2[b], Tr-3[c], Tr-4[d]	1HK1	2.65	3×10^{5}	[20]
Pigment	Bilirubin	L-shaped pocket in sub-domain IB	2VUE	2.42	9.5×10^{7}	[21]
Metal	Au (I)	Free Cys34 thiol	^{1}H and ^{113}Cd NMR Investigations		N.F	[22]
	Ag (I)				N.F	
	Cu (II)	N-terminal Asp1, Ala2, and His3			1.5×10^{11}	[23]
	Co (II)				6.5×10^{3}	
Exogenous ligands						
Anesthetic drug	Halothane	IIA-IIB (FA6)	1E7B	2.38	N.D	[24]
Anticoagulant drugs	Warfarin	Drug site I (Subdomain IIA)	2BXD	3.05	3.4×10^{5}	[25]
Anti-inflammatory drugs	Ibuprofen	Drug site II	2BXG	2.70	2.7×10^{6}	[25]
Benzodiazepine drugs	Diazepam	Drug site II	2BXF	2.95	3.8×10^{5}	[1]

[a]Tr-1 largely corresponds to FA7. The partial occupancy of this site does not allow to say that it matches with fatty acid binding site.

[b]Tr-2 largely corresponds to FA3–FA4. The partial occupancy of these sites do not allow to say that they matches with fatty acid binding sites.

[c]Tr-3 largely corresponds to FA5. The partial occupancy of this site does not allow to say that this site matches with fatty acid binding site.

[d]Tr-4 is located in the proximity of FA5.

[e]*K*a are affinity association constants (Scatchard constants) obtained at pH 7.4.

[f] N.D., not determined. N.F., not found

I.2.3 - Functions features

There are several transport proteins in blood plasma but albumin is the only able to bind a wide diversity of ligands reversibly with high affinity. Albumin has a strong negative charge, but binds weakly and reversibly to both cations and anions [26].

The regulation of both colloid osmotic pressure and capillary membrane permeability, ligand binding and transport, free radical scavenging, as well as anti-oxidant and circulatory protective properties are the physiological functions recognized for HSA [27]. HSA is also used as an excipient for vaccines or therapeutic protein drugs and as a cell culture medium supplement in the production of vaccines and pharmaceuticals[28]. Moreover, HSA could act as a carrier of O_2, drugs and fusion peptides [1].

Human Serum Albumin is an important biomarker of many diseases, such as cancer, rheumatoid arthritis, and ischemia. Furthermore, HSA is largely used clinically to treat several diseases, like hypovolemia, shock, burns, trauma, hemorrhage, cardiopulmonary bypass, acute respiratory distress syndrome, acute liver failure, nutrition support and many others [1].

Besides this HSA has enzymatic properties. It presents esterase, enolase and RNA-hydrolyzing activity. For example, the esterase and enolase activity of HSA is important in pharmaceutical and clinical areas because it can be used to activate pro-drugs, in fact the esterase activity of HSA could be used to convert an acetylated pro-drug into a function drug. This activity can also be used to identify potential breast tumor cells, as the intracellular albumin concentration and enolase activity, allows the differentiation between benign and malignant breast tumors [29].

HSA also plays a role in biotechnological applications for example in the development of implantable biomaterials, surgical adhesives and sealants, biochromatography, ligand trapping, and fusion proteins [1][30].

I.3 - Albumin receptors

Numerous receptors have been reported for HSA, namely glycoprotein gp18, gp30 and gp60 (albodin), the secreted protein acidic and rich in cysteine (SPARC- also called osteonectin) and neonatal Fc receptor. These interactions control the fate of albumin, its degradation, transport across or exchange between compartments, salvage and recycling [14].

I.3.1 Glycoprotein 18, gp 30 and gp 60

Glycoprotein 18 and glycoprotein 30 are present in cultured fibroblasts, smooth muscle cells and endothelial cells and are found in the heart, lung, muscle, kidney, brain, pancreas and liver. This glycoprotein resembles scavenger proteins and can mediate the high affinity binding endocytosis and degradation of modified albumins but not native albumins [14].

Glycoprotein 60 is a central component of albumin transcytosis. This protein is present in the vascular continuous endothelium and alveolar epithelium. In opposite with the other glycoproteins, the gp 60 albumin receptor binds native albumin and not chemically modified albumins [14].

I.3.2 Human neonatal Fc receptor – FcRn

Serum albumin is a natural carrier protein possessing multiple ligand binding sites and a plasma half-life ~19 days [31]. The neonatal Fc receptor (FcRn) serves to rescue, IgG and albumin, from degradation, and thereby prolong their half-lives [32] this promotes HSA as a highly attractive drug delivery technology [31]. These two ligands bind simultaneously structurally separate binding sites on FcRn, in a non-cooperative manner [33].

This receptor is widely distributed in many tissues and cell types including vascular, renal and brain endothelia [32]. FcRn is a heterochimeric receptor, comprising of a major histocompatibility class I (MHC-I) heavy α-chain and a noncovalent associated β2-microglobulin (β2m) light [33] (Fig 2A). IgG and albumin enter after pinocytosis in intracellular endosomes

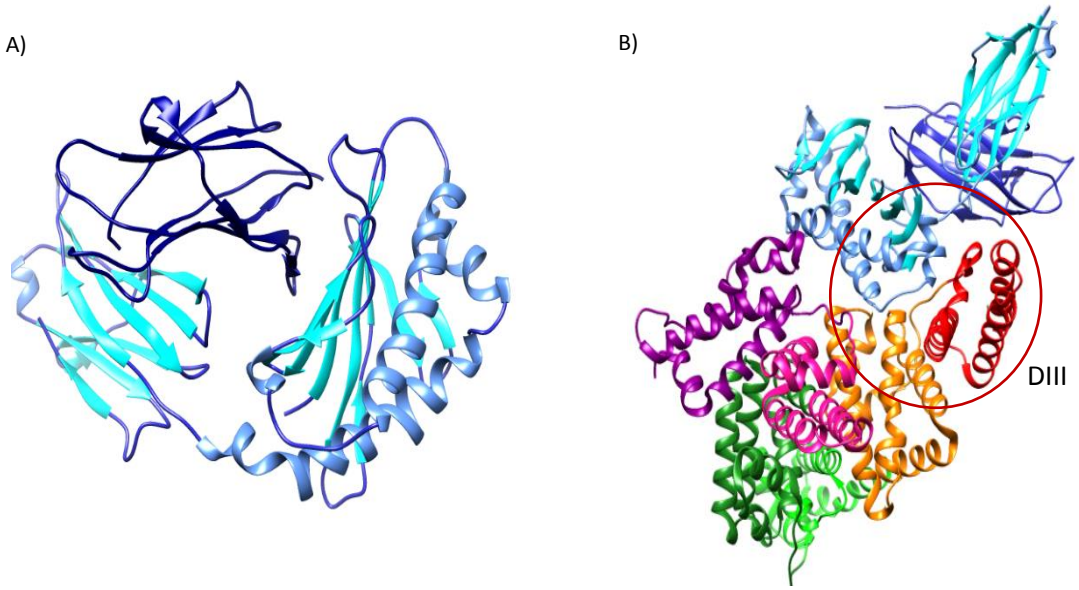

Figure 2 - Structure of the Human Neonatal Fc Receptor. Representation in ribbon models**. A**) Determined by x-ray crystallography with resolution 2.6 Å. The cornflower blue and cyan represents the heavy chain (helix in cornflower blue and strand in cyan) and dark blue correspond to β2-microglobulin. PDB code (3M1B).**B)** Determined by x-ray crystallography with resolution 3 Å. Structure of the interaction between heavy α-chain(cornflower blue) and domain III of HSA (red circle). (PDB code:4N0F). Image built in UCSF Chimera software.

where they bind FcRn as a consequence of the low pH. This is mediated by histidine residues, mainly histidine 166 located within the α2 domain of the FcRn heavy chain that become positively charged at acidic pH and are located to the elbow region of the IgG Fc, the DIII of albumin (Fig 2B) [32]. The ternary complex is recycled to the cell surface where exposure to the

physiological pH of the blood triggers release. Thus, FcRn rescues both its ligands from intracellular degradation, whereas proteins that do not bind the receptor are directed to lysosomes (Fig 3) [33].

Two distinct diseases may be manifestations of FcRn malfunction, the familial hypercatabolic hypoproteinemia (FHH) that results from a deficiency of FcRn due to a mutant β2-microglobulin (β2m) gene and myotonic dystrophy (DM), which shows hypercatabolism and plasma deficiency of only IgG but not albumin [34] .

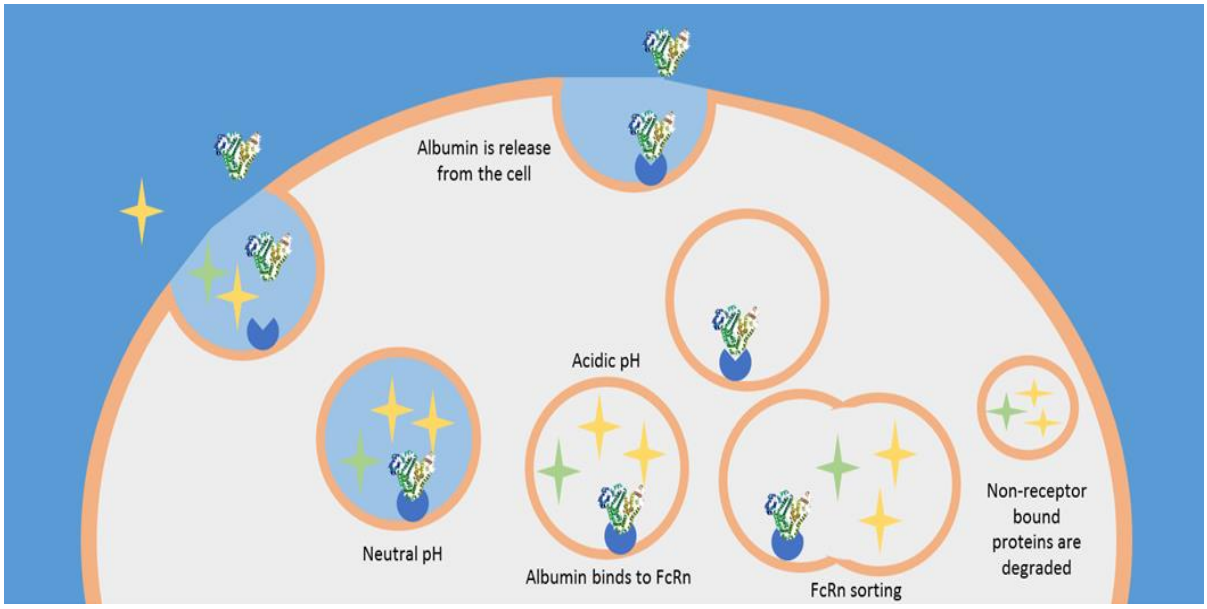

Figure 3 - FcRn mediated, pH-dependent, albumin recycling. Extracellular albumin (gold heart-shaped protein)) is pinocytosed along with a range of other plasma constituents at neutral pH (blue shading). Upon acidification of the vesicle (gray shading) albumin binds to the integral FcRn membrane protein (purple molecule). Non-FcRn interacting proteins and plasma constituents are sorted to the lysosome for degradation while vesicles containing functional albumin–FcRn complexes are recycled back to the plasma membrane cell surface where they are exposed to neutral pH of the plasma and the albumin is released back into the circulation [15].

In 2010, approximately 200 biologics were approved for therapeutic applications, where monoclonal antibodies and vaccines account for more than 60%. In addition hormones, cytokines, grow factors, coagulation factors, enzymes, fusion proteins are developed and used as therapeutics [35]. Many of these protein drugs possess a molecular mass below 50 kDa and a rather short terminal half-life in the range of minutes to hours. In this cases are developed and implemented of half-life extension strategies to prolong circulation of these in the blood and thus to improve administration and pharmacokinetic as well as pharmacodynamics properties[35].

I.4 - Albumin binders

I.4.1 - Natural binders

Many gram-positive bacteria express surface proteins able to bind with several human serum proteins that include immunoglobulin, serum albumin, fibrinogen, fibronectin, haptoglobulin and aggregated β_2- macroglobulin [36].

Expression of albumin-binding proteins has been shown to promote bacterial growth and virulence. The bacterial species that express albumin-binding domains are usually part of the normal human flora and they are opportunistic pathogens[37].

Non-covalent association to albumin can be used to extend the half-life of drugs, which has been investigated using several albumin-binding it is important to consider remember that FcRn-binding site on albumin is located in domain III with the help of domain I so not interfere with binding [37].

There are several albumin-binding domains (ABDs) that bind to HSA as protein G [38], protein PAB [39], protein MAG [40] , protein ZAG [41] and protein H [36] (Table 2).

Table 2 - List of several albumin-binding domains that binds to HSA

ABDs	Organism	Size (Aas)[a]	References
Protein PAB	*Peptostreptococcus magnus*	45	[39]
Protein G	*Group C or G Streptococcal*	46	[38]
Protein H	*Strepgococcus pyogenes*	112	[36]
Protein MAG	*Streptococcus dysgalactiae*	60	[40]
Protein ZAG	*Streptococcus zooepidemicus*	52	[41]

[a]Size Aas- Aminoacids

I.4.2 - Synthetic binders

The designed ligands are generally intended to improve the performance of natural binding ligands (such as Protein A) [42].

The design and development of engineered pseudobio specific affinity ligands with desired and improved properties are possible due to the progress in modecular modeling, genetic engineering and natural and synthetic combinatorial methodologies. These ligands include biological (peptides and engineered protein domains) and synthetic (design dyes) molecules [42].

Human serum albumin is typically isolated from human plasma by Cohn classical blood fractionation but Cohn's method concerns precipitation of proteins using ethanol with varying pH, ionic strength and temperature. This technique is not highly specific and can give proteins, partially desnaturated proteins. Biomimetic dye-ligands offer advantages over biological ligands in terms of economy, ease of immobilization, stability and high adsorption capacity. Cibacron Blue F3GA is a group specific affinity dye that possess a high affinity to albumin [43].

General or group-specific affinity ligands, exhibit wide spectrum of interaction with protein molecules, so their selectivity is reduced and they can be used in different purification cases. The next table summarises the most important types of such affinity ligands [44].

Table 3 - Most important differences between Biological and Synthetic affinity ligands

Criterion	Dye-ligand absorbent	Biological-ligand absorbent
Economy	Inexpensive, commodity chemicals	Expensive (e.g antibodies)
Synthesis	Easy	Often lengthy routes
Stability	Chemically and biologically stable	Biodegradable and chemically labile
Capacity/ligand utilization	High	Moderate to low
Specificity	Moderate (dyes) to high(biomimetic dyes)	High to moderate
Scaling-up	Large-scale potential	Limited application

There are several different approaches to ligand design and synthesis that form the basis of the technology used today for commercial development of third-generation adsorbents, including: a) optimization of existing ligands (analog synthesis); b) rational design (computer modeling of ligand structures); c) systematic screening of ligand arrays; d) rational design combined with library construction and screening [45]

Chapter 2.
BACKGROUND AND AIM OF THE WORK

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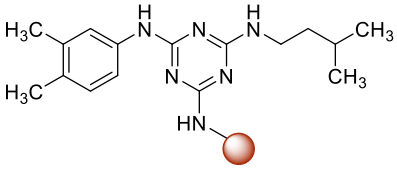
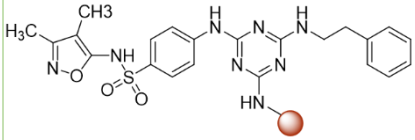
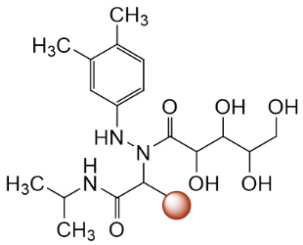
Serum albumin is a natural carrier protein possessing multiple ligand binding sites. It is an important biomarker of many diseases, such as cancer, rheumatoid arthritis, and ischemia and is largely used clinically to treat several diseases.

For this reason, the development of ligands with affinity for HSA may be an effective strategy for improving the pharmacokinetic properties of drugs or biodrugs and also for HSA purification, which posteriorly can be used for diagnostic and clinical purposes. Within this context, the aim of this research project is the development of biological and synthetic affinity ligands against HSA.

For this purpose, two libraries of synthetic affinity ligands based on Triazine reaction and other on Ugi reaction have been previously synthesized and screened in our group. Three ligands have been selected as the most promising, namely A3A2 and A6A5 from Triazine library, and C7A3 from Ugi reaction (Table4). These ligands show selectivity for HSA in comparison with polyclonal human IgG.

Table 4 - Affinity ligands from Triazine reaction (A3A2,A6A5) and Ugi reaction (C7A3

A3A2	A6A5	C7A3
		

The biological affinity ligands were based on small engineered WW domains previously evolved by ribosome display to bind to HSA and human IgG. Three proteins were selected for their specificity to bind HSA and IgG.

Table 5 - Comparison between the fusions WW with GFP, selected by ribosome display. In green are the amino acids that take part in loops and that were randomized. In bold are the amino acids that are in limits of the loop.

WW prototype	Amino acid sequence	Target
H4	SMGLPPGWDEYK**T**RLWYW**G**KTYYYN**H**WTDN**K**TSTWTDPRMSS	HSA
H18	SMGLPPGWDEYK**T**FWCLS**G**KTYYYN**H**WWWS**K**TSTWTDPRMSS	HSA
I8	SMGLPPGWDEYK**T**WLWAL**G**KTYYYN**H**GCRR**K**TSTWTDPRMSS	IgG

Based on these previous results, and in view of the importance of HSA for several biotechnological and biomedical areas, this dissertation had four main goals:

Synthetic affinity ligands

(i) Optimization of binding and elution conditions for purification of albumin from plasma;

(ii) Study of static partition equilibrium for the determination of binding constant;

Biological affinity ligands

(i) Production and purification of GFP, WWH18_GFP, WWH4_GFP and WWI8_GFP:

(ii) Study of interaction between WW prototypes and HSA by ELISA assays.

Approached for the synthetics ligands

(i) Choice of elution buffers for each ligand

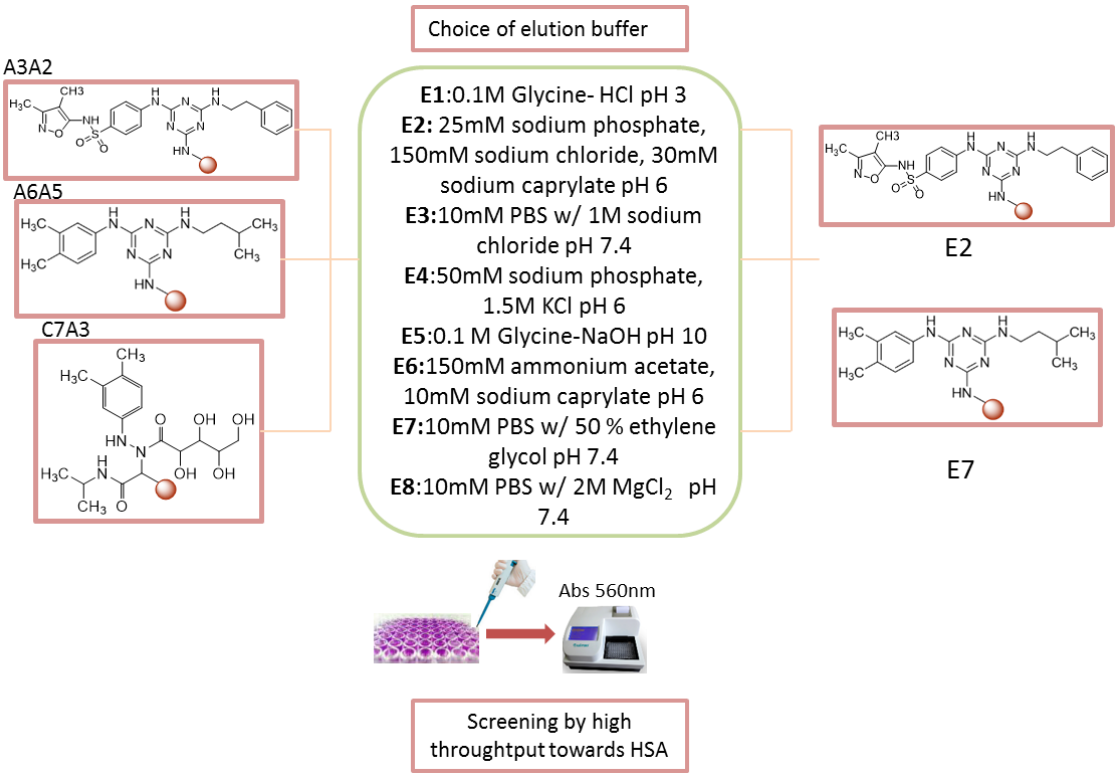


Figure 4 – Schematic representation of the research work followed in this work.

(ii) Optimization of binding and elution buffer and screening against plasma

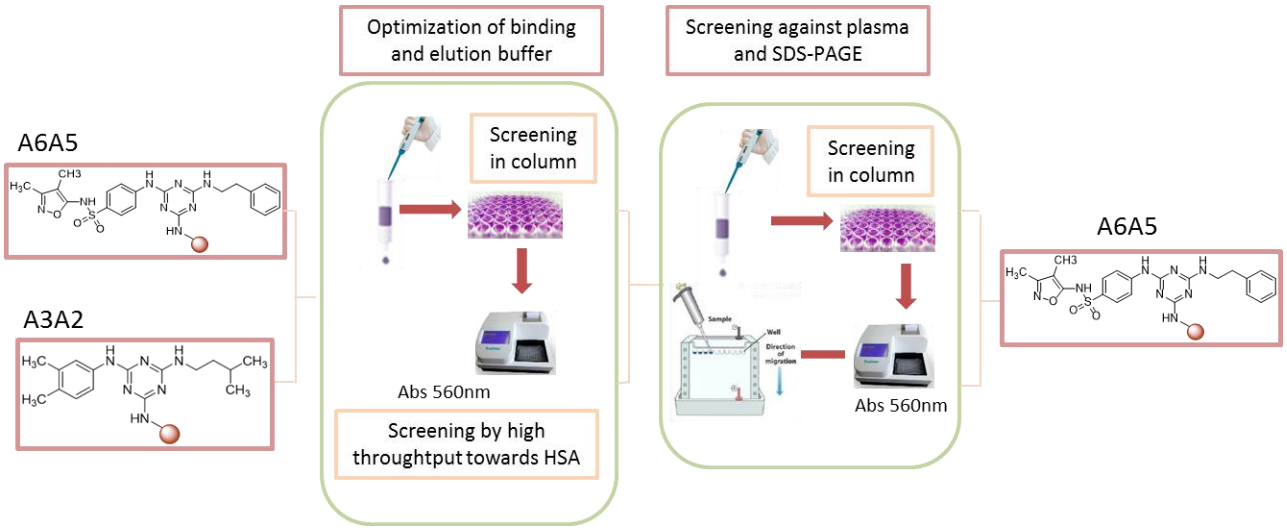


Figure 5 - Schematic representation of the research work followed in this work.

(iii) Realization of static partition equilibrium studies for the determination of binding constants and dynamic binding capacity.

Approached for the biological ligands

(i) Production, purification of GFP, WWH4, WWH18, WWI8

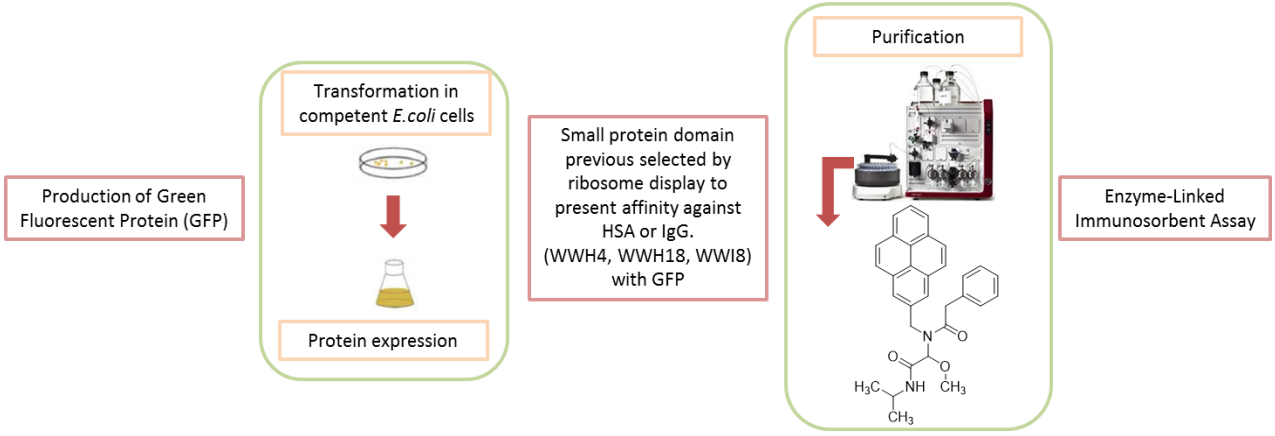


Figure 6 - Schematic representation of the research work followed in this work.

Chapter 3.

PURIFICATION OF HSA WITH SYNTHETIC AFFINITY LIGANDS

The aim of this Chapter was find the best ligand with the capacity to purify HSA from the plasma. First was evaluated the selectivity of the synthetic ligands based on Ugi and Triazine reactions for HSA binding. The binding and elution conditions were optimized and the best result was tested against plasma. To confirm the results was performed SDS-PAGE. The binding constants Ka(M^{-1}) and Qmax (mg protein bound/g support) were realized using Langmuir model and Scatchard plot Afterwards, Dynamic Binding Capacity was performed.

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III.1 - Introduction

Human serum albumin (HSA) is the most abundant protein in the circulatory system. Because of its availability, low cost, stability, and unusual ligand binding properties, serum albumin has been one of the most extensively studied and applied proteins in biochemistry [12].

It is an important biomarker of many diseases, such as cancer, rheumatoid arthritis, and ischemia. Furthermore, HSA is largely used clinically to treat several diseases, like hypovolemia, shock, burns, trauma, hemorrhage, cardiopulmonary bypass, acute respiratory distress syndrome, acute liver failure, nutrition support and many others [1].

For this purpose, the ideal product is a monomeric albumin with high purity, free from contaminations with other plasma proteins, endotoxins and metal ions, such impurities appear influence the tolerability of albumin infusions [46]. Therefore, it is necessary to develop purification methods selective for HSA. In 2010, 78 fractionation plants were operating in the world, 25 of which were in China, 26 in Europe and 8 in the United States of America. The world plasma fractionation capacity was about 48.4 million liters. Annually from more than 34 million liters of plasma, are produced more than 500 tons of HSA and more than 40 tons of intravenous immunoglobulins (IVIG). The average HSA consumption in the developed world is 200-400 kg per million population [4].

At industrial level there are several techniques established for the purification of albumin from serum or plasma, such as ethyl alcohol fractionation denominated Cohn method, heat shock and chromatography [5]. The fractionation developed by Cohn and colleagues is a process based in the difference of solubility of albumin and other plasma proteins at different protein concentration, pH, temperature, ionic strength and ethanol concentration. With this method a large volume of fractionation plasma is obtained and the proteins acquire are safe for therapeutic use. Nevertheless, for this fractionation it is necessary a high quality starting material, refrigeration system and exist risk of protein denaturation [6].Another method is thermal shock, where plasma proteins are exposed to high temperature (60 ^{o}C) to inactivate potential pathogens, at this temperature HSA does not denature instead other serum proteins are denatured and rendered insoluble under such conditions. Normally, the purity of a heat shock HSA is greater than 98% [5]. Although the traditional plasma fractionation techniques are based on ethyl alcohol fractionation, more and more, plasma fractionation technologies include chromatographic step [46].Of all purification methods, chromatography may be the best candidate for minimizing process generated albumin heterogeneity.

Affinity purification involves the separation of molecules in solution (mobile phase) based on differences in binding interaction with a ligand that is immobilized to a stationary material (solid phase). The solid phase in affinity purification is a support or matrix material that a biospecific ligand may be covalently attached [47].

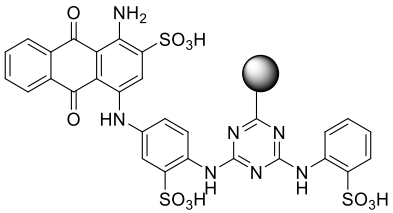
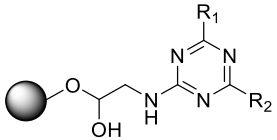
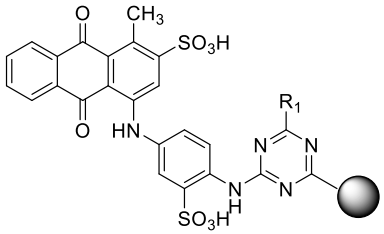
Affinity chromatography is the most widely employed method for the purification of proteins, and based on highly specific molecular recognition. Dye-ligand chromatography offer some vantages for protein purification compared with other forms of affinity chromatography, such as is possible storage of matrix without loss of activity, are reusable withstands cleaning and sterilization and are easy of immobilization[48].

On the market, currently, exist kits for HSA purification (Table 6) based on affinity chromatography. The biomimetic monocholoro or dicholoro triazinyl dyes are able to bind most type of proteins [48]. One of the examples of this type of synthetic ligand in affinity chromatography for isolation and purification of HSA is a Cibacron Blue. However, the heterogeneity and low purity of the Cibacron dye can affect its use in the introduction of the purification of HSA at production scale for clinical use [49].

The objective of the work are focused on proteins that bind to the domain II of HSA. The affinity ligand mimics the amino acids from protein PAB present in the interaction between this protein and HSA (Annex I).

In this Chapter was studied three different ligands from different libraries and the objective was find the best ligand that have the capacity the purified HSA from the plasma.

Table 6- Kits currently available on the market based on affinity chromatography. The structures were design in Chimera software

NAME	Structure	Resin	Dynamic Binding Capacity	Cost (USD)	Binding Buffer	Elution Buffer	REF.
Cibacron blue 3G-A		Synthetic ligand based on Triazine scaffold immobilized in highly cross-linked agarose	≤ 18 mg HSA/mL	228.99/g	0.05 M citric acid, 0.1M Na2HPO4 pH7	0.05 M KH2PO4, 1.5 M KCl pH 7	Sigma-Aldrich GE-Healthcare
Capto blue Sepharose		Highly cross-linked agarose	24 mg HSA/mL	8.5/mL	20 mM citric acid pH 5.5	50 mM sodium phosphate, 2M NaCl pH 7	GE-Healthcare
Mimetic Blue 1A6XL		Synthetic ligand based on Triazine scaffold immobilized in highly cross-linked agarose	7 mg/mL	12.60/mL	25 mM MOPS buffer pH 6	50 mM sodium phosphate, 2M NaCl pH8	Prometic Biosciences
AlbuPure	N/A	Ligand developed by Novozymes Highly cross-linked	> 35 g/L	15.40/mL	50 mM sodium citrate pH 5.5	50 Mm ammonium acetate, 10 Mm sodium caprylate pH7 or 50 Mm sodium carbonate pH 10	Prometic Biosciences
Affibody	N/A (possible to re-use for 300 times)	Affibody anti-HSA immobilized in SulfoLink® Coupling Gel	160 µL serum/ mL of resin	2.14/µg	25 mM Tris-HCl, 1mM EDTA, 200 mM NaCl, 0.05% tween 20, pH 8	0.5 M Acetic acid, 1M Urea, 100 mM NaCl, pH 2.8	Affibody

III.2 - Materials

III.2.1 - Chemicals

All reagents were used with a high purity and the solvents were pro-analysis.

The reagents Phenethylamine (A5), Sulfisoxazole (A6), Human Serum Albumin (HSA), IgG from human serum and Bovine Serum Albumin (BSA) were purchased from Sigma- Aldrich (Portugal).

Acetone, Epichlorohydrin, Cyanuric chloride, Isopropyl isocyanide, Phenol, Sodium bicarbonate (NaHCO3), Sodium thiosulfate (Na2S2O3), Glycine, Sodium Chloride (NaCl) and QuantiProTM BCA Assay Kit were acquired from Sigma-Aldrich (Portugal). Methanol (MeOH) and Ammonium hydroxide solution (NH4OH) were obtained from Roth. Dimethylformamide (DMF), Methanol (MeOH), Di- Sodium-hydrogen Phosphate 2-hydrate and Sodium hydroxide (NaOH) were purchased from VWR (Portugal). Ethanol absolute PA, Sodium-di-hydrogen Phosphate 1-hydrate, hydrochloric acid 37% (HCl) were acquired from Panreac (Spain).

III.2.2 - Chromatographic materials

Agarose SepharoseTM CL-6B was acquired from GE Healthcare; Captiva 96-well filtration block was purchased from Agilent Technologies (USA); Half-area UV-Star® 96-well microplates and 96-well transparent microplates were obtained from Greiner Bio-One (Germany) and Sarstedt (Portugal), respectively. For the screening in column was used Agilent bond Elut 3ml and Frits from Agilent Technologies (USA). For determined Dynamic Binding Capacity was used Tricorn™ empty high performance columns (GE Healthcare, Uppsala, Sweden).

III.2.3 - Buffers

One objective of this work was optimized buffers for purification of HSA for this purpose were used different buffers. The buffers used for binding were Phosphate Buffer Saline (PBS) (10mM Sodium phosphate, 150mM NaCl, pH 7.4); 0.01 M Tris-HCl, 150 mM NaCl, pH 8; 0.01 M Tris-HCl, 300 mM NaCl, pH 8; 50 mM sodium citrate pH 5; 50 mM sodium citrate, 300 mM NaCl, pH 5.

The buffers used in the elution step were 0.1M Glycine- HCl at pH 10 and 3 in this case were used Tris (hydroxylmethyl) aminomethane base at pH 9.0 for neutralization of eluted samples, were used 25 mM sodium phosphate, 150 mM sodium chloride, 30 mM sodium caprylate, pH 6; 150 mM ammonium acetate, 10 mM sodium caprylate pH 6, 10mM sodium phosphate, 10M NaCl, pH 7.4; 50 mM sodium phosphate, 1.5 M KCl, pH 6; 10mM Sodium phosphate, 150 mM NaCl, 50 % ethylene glycol pH 7.4; 10mM Sodium phosphate, 150 mM NaCl,

2M $MgCl_2$, pH 7.4; 25 mM sodium phosphate pH 6; 25 mM sodium phosphate, 30 mM sodium caprylate pH 6. In the end was used the Regeneration Buffer (0.1M NaOH in 30% isopropanol)

III.2.4 - Equipment

The synthesis of the synthetic ligands on solid-phase based on Triazine and Ugi reaction were carried out in Incubator ZKA KS4000i (VWR). The screening was performed using a LMC 3000 laboratory centrifuge from Biosan and used Microplate reader TECAN Infinite F200 from Thermo Scientific (Portugal). For determined Dynamic Binding Capacity was used AKTA pure (GE Healthcare)

III.3 - Methods

III.3.1 - Preparation of ligands

III.3.1.1 - Epoxy-activation of agarose

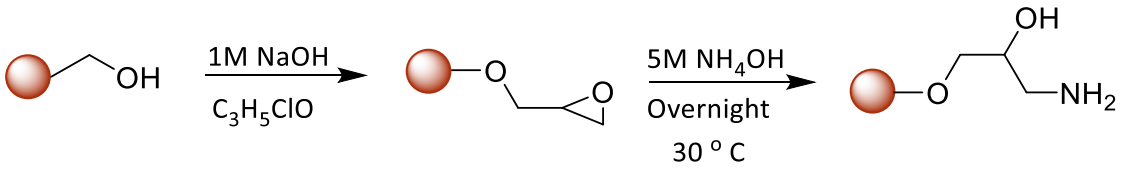


Figure 7- Reaction squeme of Epoxy-activation of agarose and functionalization with amine groups.

This is the first step of the synthesis of ligands and start with the wash of Sepharose$_{TM}$ CL-6B (120 g) with distilled water (10 × volume of resin's weight) and filtered with the aid of a filter under vacuum. After, the agarose was resuspended in 120 mL of distilled water and 4.8 mL of 10 M NaOH (0.04 mL per gram moist agarose). Then, this suspension was incubated for 30 minutes at 30 º C with orbital shaking at 200 rpm.

Posteriorly, 6.84 mL Epichlorohydrin (0.072 mL per gram moist agarose) was added and incubated for 3 hours at 36ºC in orbital at 200 rpm. After this time, the resin was washed with distilled water (10 × resin volume). Rapidly, the extension of epoxy-activation was determined through the quantification of hydroxyl groups (OH-) exposed by the opening of the epoxy ring. In this test 3 mL Sodium Thiosulfate was added to 1g of epoxy-activated agarose and incubated after agitated for 20 minutes at room temperature. Passed this time the OH- groups were titrated with 0.1 M HCl until pH arrives to pH 7. The volume added correspond to the number of hydroxyl groups (20 µmoles per 200µl HCl added). Normally, the values of the epoxyativation

are between 21 ±1 µmol of epoxy/g of agarose. In this case, the epoxy-activation obtained was 22 µmol/g of agarose.

III.3.1.2 - Functionalization of epoxyactivated agarose with amine groups

For this step 85g of epoxy-activated agarose was resuspended in 127.5 mL of 5M of Ammonium hydroxide (1.5 mL per gram of moist resin) and incubated overnight at 40ºC with agitation at 200 rpm. Then, aminated agarose was washed with distilled water (10 × resin volume). Afterwards, was performed Kaiser test, for confirm the presence of the amines in agarose. The test allows to quantify the free amines (-NH_2) present in a given sample and is based on the reaction of ninhydrin with primary amines, which gives a characteristic dark blue color. In this test 50µl of 5% ninhydrin in ethanol (w/v), 50µl of 80% phenol in ethanol (w/v) and 50µl of 2% 0.001M potassium cyanide in pyridine (v/v) were added to suspension of 0.1g of aminated agarose with 0.9mL of distilled water and heated in a water bath at 100°C for 5 minutes. In order to obtain the parameters that allow to determine the concentration of primary amines in each sample, one calibration line to standard glycine solutions (0-5 µmol/mL) versus their absorbance at 560 nm, was performed, in triplicates. Was obtained the following curve $y = 3.286\,x - 0.04$, with R^2= 0.99, where concludes that exist 12.02 µmol NH_2/g moist agarose.

III.3.1.3 - Functionalization of epoxyactivated agarose with aldehyde groups

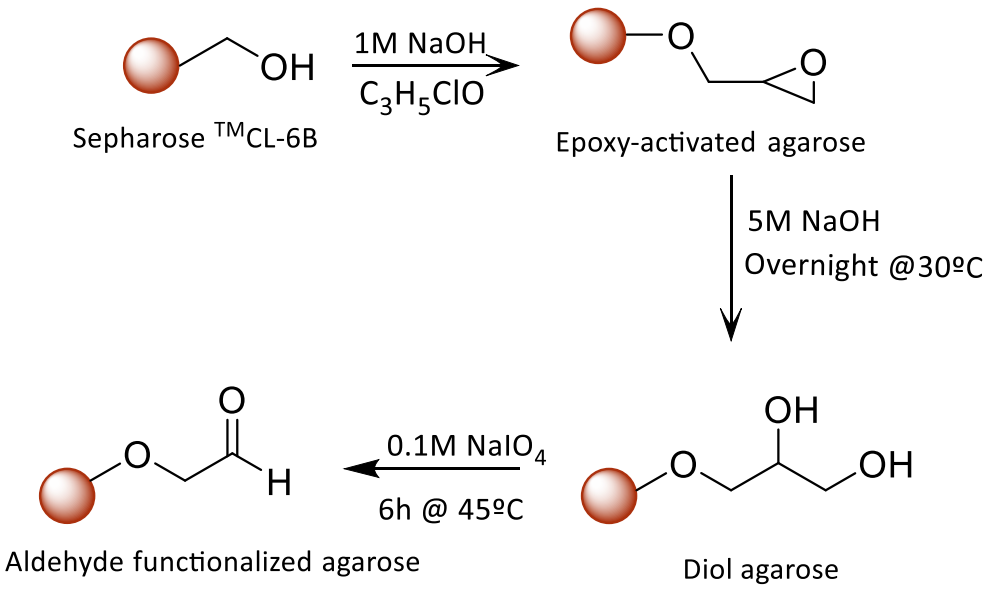


Figure 8- Reaction squeme of Epoxy-activation agarose and functionalization with aldehyde groups.

For this functionalization 35 g of epoxy-activated agarose was resuspended in 35 mL of 5M of NaOH (1 mL/g moist agarose) and incubated overnight at 30°C with orbital shaking at 200

rpm. In the end, resin was washed with distilled water in a filter under vacuum and resuspended in 35mL of 0.1M of Sodium periodate (1 mL/g moist agarose) and incubated at 45°C at 200 rpm for 6 hours. Afterwards, the functionalized agarose was washed with distilled water (10 × resin volume).

III.3.1.4 - Synthesis of the solid phase combinatorial library based on the Triazine reaction

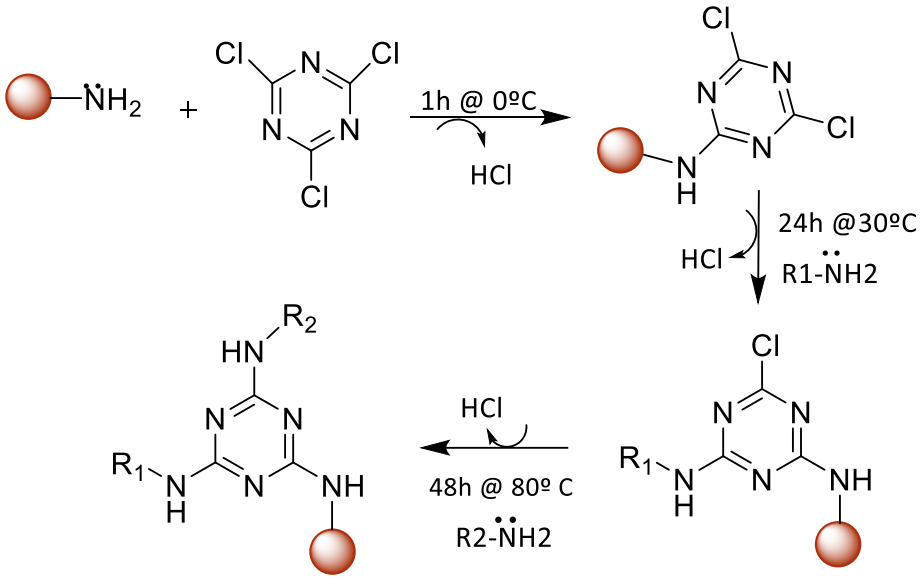


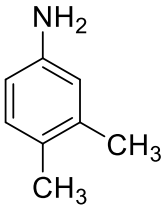
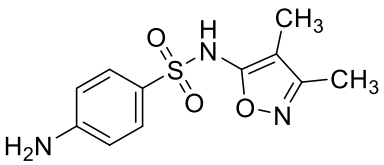
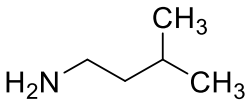
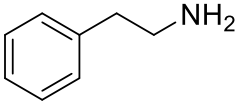
Figure 9- Reaction squeme of synthesis of the solid phase combinatorial library based on Triazine reaction

For start this stage aminated agarose was washed with distilled water. Then was resuspended in a cold solution of 50% (v/v) acetone/ dd water with 1 molar eq. of $NaHCO_3$ relative to epoxy (1 mL/ g moist agarose). After, was added Cyanuric chloride (5 eq. molar excess relative to epoxy content) dissolved in acetone (8.6 mL/g cyanuric chloride) to the resin, followed by an incubation for 1h at 0°C at 200 rpm.

After incubation, the cyanuric chloride-functionalized resin was washed with acetone (2x resin volume), 50% (v/v) acetone/dd water (3x resin volume) and dd water (5x resin volume). After this, the resin was divided in two bottles with different amines. Amines A3 and A6 were added to each bottle (2 molar eq. of each relative to epoxy) and incubated for 24h at 30°C with agitation (200 rpm). Afterwards, the ligands were washed with the solvent in which each amine was dissolved (5 × resin volume) and distilled water (5 × resin volume).

Subsequently, amines A2 and A5 were added to each bottle (5 molar eq. of each amine relative to epoxy)(Table 7), and incubated for 48h at 80°C in orbital shaker (200 rpm). In the end, the ligands were washed with the solvent in which each amine was solubilized (5 × resin volume), 0.1 M hydrochloric acid (HCl) (1 × resin volume), distilled water (1 × resin volume), 0.1 M NaOH in 30% isopropanol (v/v) (regeneration buffer; 1 × resin volume), and distilled water (5 × resin volume). The amines were dissolved in 50% (v/v) DMF/ distilled water. In this reaction, all the amines were firstly dissolved in 50%DMF/distilled water, before add to the bottles. In addition, it was added 1M Sodium bicarbonate (1 molar eq. relative to epoxy) in each amine.

Table 7-Amines used for the synthesis of Triazine

R1	**A3** (3,4 Dimethyalanine)	**A6** (Sulfixazole)
		
R2	**A2** (Isopenthylamine)	**A5** (Phenethylamine)
		

The ligands were then distributed by 96 wells of a 96-well filter plate (0.25 g/ well), and the solvent was left to drain by gravity (Fig.10), the rest of ligand was stored at 4°C with 20% ethanol.

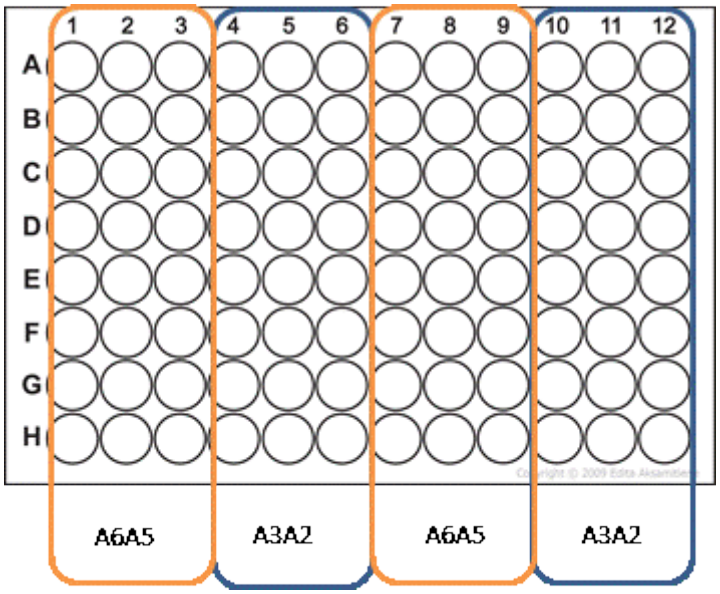


Figure 10- Illustrative image of the 96-well filtration block used for the synthesis of the ligands based on Triazine reaction. In orange is represented the affinity ligand A6A5 and blue the affinity ligand

III.3.1.5 - Synthesis of the solid phase combinatorial library based on the Ugi reaction

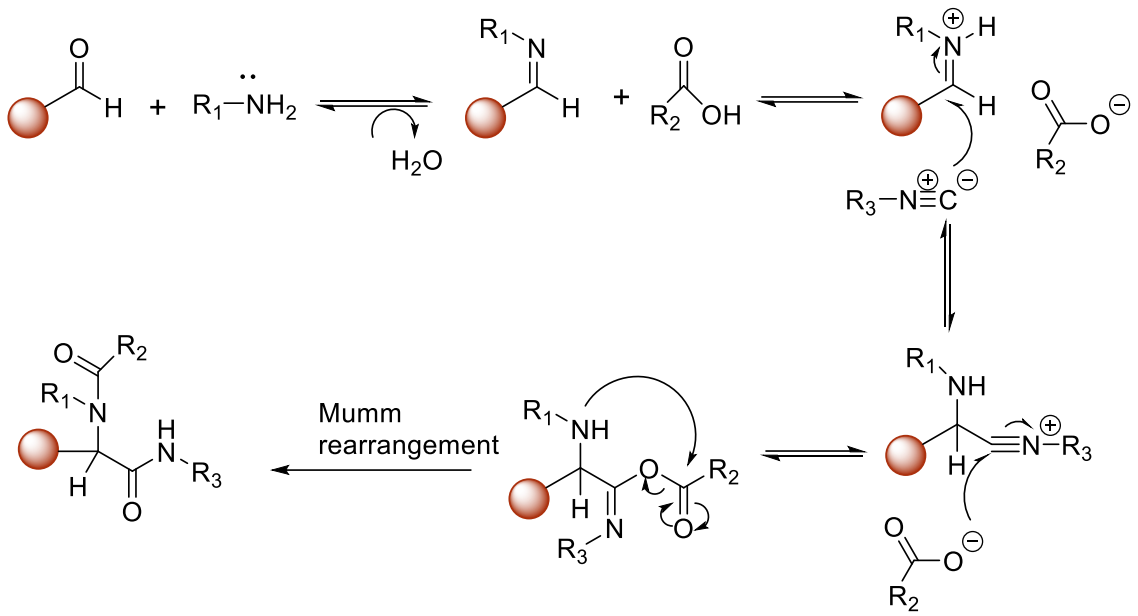
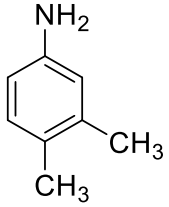
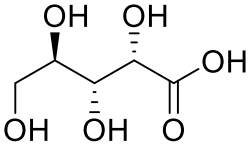


Figure 11-Reaction squeme of synthesis of the solid phase combinatorial library based on Ugi reaction

In the Ugi reaction the agarose functionalized with aldehyde groups was washed with 20% of methanol, from 0% (v/v) to 100% (v/v) methanol. After was added the amine A3 to the bottle (5 molar eq. relative to epoxy) and incubated for 2h at 60°C with orbital at 200 rpm. After this, carboxylic acid compound C7 (5 molar eq. relative to epoxy) and Isopropyl isocyanide (5 molar eq. relative to epoxy)(Table 8) were added and the agarose was incubated for 48h at 60°C in orbital at 200 rpm.In the end of the reaction, the ligand was washed with 1ml of the following reagents: 100% methanol (v/v), 50% methanol (v/v), 50% DMF/distilled water (v/v), distilled water, 0.1M HCl, distilled water, 0.2M NaOH in 50% isopropanol (v/v), 2x distilled water and 20% ethanol (v/v) for store at 4ºC. In this reaction, the amine, A3, was dissolved in methanol and carboxylic acid, C7, was dissolved in 50% methanol/distilled water (v/v).

Table 8-Compounds used for the synthesis of Ugi reaction.

R1	R2
A3 (3,4 Dimethyalanine)	**C7** (Arabic acid)
	

III.3.1.6 - Regeneration and equilibration of the combinatorial libraries

The regeneration and equilibration of the ligands were necessary before the realization of screenings. The plate and bottle with the affinity ligands were washed with 0.1 M NaOH in 30% isopropanol (regeneration buffer) followed by distilled water (3 cycles of washes, 0.75 mL/well). After this process, the equilibration was realized with the addition of the 10mM Phosphate Buffer, 150mM NaCl at pH 7.4, the binding buffer (15 × 0.75mL). In the last wash, 100µl were collected in a half-area UV-Star 96-Well microplate, and read at 280nm. The last wash was performed when the absorbance values were lower than 0.005. Then, the ligands was prepared for screening or was added 20% ethanol (v/v) (0.75ml/well) for store at 4ºC.

III.3.2 - Screenings

III.3.2.1 - Screening and elution of the Triazine ligands

For this procedure, a solution of pure HSA and IgG in Phosphate Buffer at pH 7.4 (0.25 mg/mL) was prepared. After, 0.25ml of the solutions were loaded to each well of the ligands (Figure12). The block was incubated with the loading for 1 hour at 25ºC with agitation (200rpm). Posteriorly, the flow-through and the washes were collected with its respective binding buffer (5x 0.25ml). Both were collected in 96-well transparent microplates by centrifugation at 600rpm during 20 seconds.

After the screening, different elution buffers were added (0.25 mL) in each well (Fig.6) and collected in 1.5 ml microcentrifuge tubes (5 cycles of elution). Then, were collected in 96-well transparent microplates by centrifugation at 600rpm during 20 seconds. To avoid denaturation of the eluted proteins, 1M Tris-base pH 9.0 was added to the E1 (0.1 M Glycine-HCl pH3) when collected the elution fractions, to adjust the pH value to 7 µL.

After the recovery, the total protein present in the samples was quantified by using the QuantiProTM BCA assay kit, for the elution E7 and E8 was realized the change buffer for PBS pH 7.4 before the realization of the quantification described in III.3.2.3. With this kit, 150µl of the QuantiProTM BCA reagent was added to 150µl of each sample added and incubated at 60ºC for 1 hour. The absorbance of the samples was then determined by measured at OD in the microplate reader at 560nm, with a calibration curve using solutions with known protein concentration BSA with different binding and elution buffers from 0 to 200µg/ml. In each samples of the calibration line E1 was added 7 µL 1M Tris (hydroxylmethyl) aminomethane at pH 9.0.

After the screening, the plate was washed with 1ml of the following solvents: 0.1M glycine- NaOH at pH 9.0 in 50% Ethileno glycol (v/v) (1×); 0.1M Glycine – NaOH at pH 11 (1×); distilled water (1×); 0.1M HCl (2×); distilled water (1×); 0.1M Glycine at pH 2.5 (1×); 0.2M NaOH in 50% isopropanol (v/v) (2×); distilled water (1×); Phosphate buffer saline in 1M NaCl (1×); 0,1 M NaOH in 30% isopropanol (1×). Then, the plate was stored at 4°C with 20% ethanol.

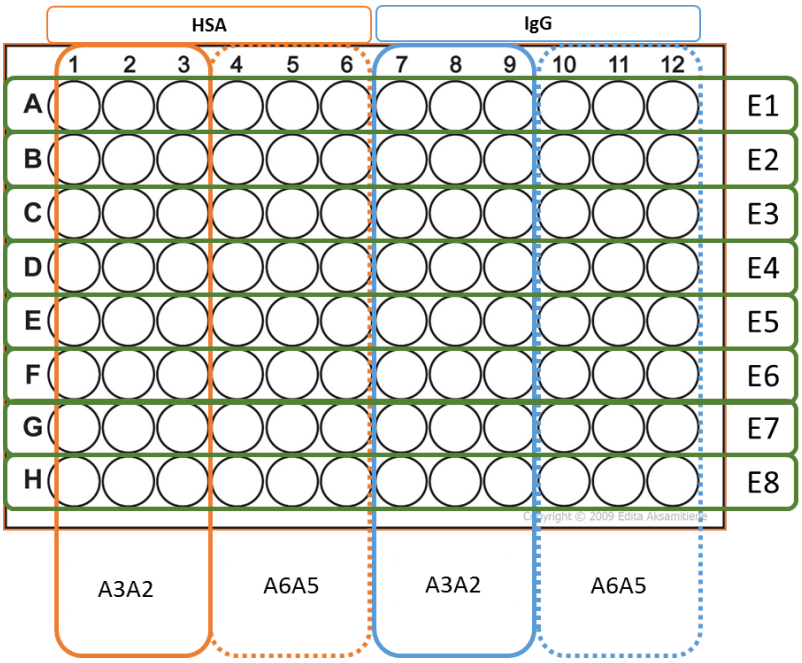


Figure 12 - Schematic representation of the research work followed in this work.

III.3.2.2 - Screening and elution of the Ugi ligand

The procedure for the screening of the Ugi ligand was prepared according to (num), instead used plate this was realized in microcentrifuge tubes. A solution of pure HSA and IgG in Phosphate Buffer at pH 7.4 (0.25 mg/mL) was prepared. Then, 0.25ml of the solutions were loaded. The eppendorfs were incubated with the loading for 1 hour at 25ºC with agitation (200rpm) (Fig.13)

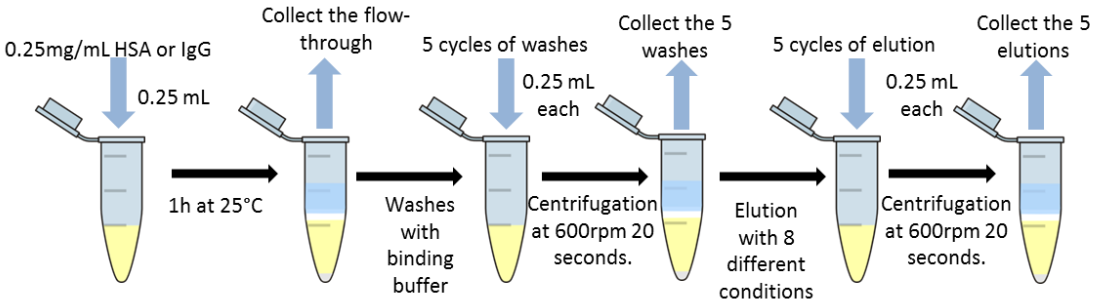


Figure 13- Procedure for screening of Ugi ligand C7A3.

Then, the flow-through and the washes were collected with its respective binding buffer (5x 0.25ml). In the end, different elution buffers were added (0.25 mL) in each microcentrifuge tube. After the recovery, the total protein present in the samples was quantified by using the QuantiProTM BCA assay kit, for the elution E7 (10mM PBS w/ 50 % ethylene glycol pH 7.4) and E8 (10mM PBS w/ 2M $MgCl_2$ pH 7.4) was realized the change buffer for PBS pH 7.4 before the realization of the quantification described in III.3.2.3 The absorbance was determined by measured at OD in the microplate reader at 560nm, with a calibration curve using solutions with known protein concentration BSA with different binding and elution buffers from 0 to 200µg/ml In each samples of the calibration line E1 was added 7 µL 1M Tris (hydroxylmethyl) aminomethane at pH 9.0

III.3.2.3 - Total protein quantification by Bicinchoninic acid assay (BCA)

The quantification of total protein performed by BCA. Firstly it was necessary to prepare a mixture with reagents A and B (A (bicinchoninic acid solution) and the reagent B (cooper (II) sulfate pentahydrate 4% Solution).

Then, were added 25 µl of sample and 200 µl of reagents in each wells. After, it is necessary incubate at 37ºC for 30 minutes. Posteriorly, it was measured in a 96-well plate reader with OD560nm (Tecan). In the end was necessary construct a calibration line for each binding or elution used solutions with known protein concentration, bovine serum albumin (BSA) from 0 to 1 mg/mL.

III.3.2.4 - Change Buffer of elution 7 and 8 for Phosphate Buffer at pH 7.4

This two buffers interfere with the process of quantification the QuantiProTM BCA assay kit, so was needed change buffer for 10mM Phosphate Buffer, 150mM NaCl at pH 7.4. For effectuated this step was used two types of membranes, *MWCO* 100 kDa for IgG (150 kDa) and *MWCO* 10 kDa (66 kDa) for HSA. Was done a joint with 100 µL of which elution (E1-E5), ending with a volume de 500 µL. The dialyses happened overnight in a bottle with for Phosphate Buffer at pH 7.4 at 4°C with agitation, during two days with the change of the for phosphate buffer. The membrane with *MWCO* 10 kDa, was needed wash with distilled water before use and the *MWCO* 100 kDa was washed with 10% ethanol for 10 min, after this 10 min washed with distilled water 20 min.

III.3.2.5 - Screening of the lead ligands in on-column format

In case of Triazine ligands after choose the elution buffer with screening in plate, was made the screening in column to find the best binding buffer. For the ligands A3A2 and A6A5

that were selected for binding HSA selectively, 0.5g of resin-functionalized was packed in a column, in duplicate. For each ligand, twelve columns were made against six binding buffers, six tested toward HSA and six tested against IgG.

After packing the columns with ligands-functionalized were washed with regeneration buffer (0.1M NaOH in 30% isopropanol) followed by distilled water (3 cycles of washes, with the maximum volume of column). Then, ligands were equilibrated with the addition of the differents binding buffers (**B1**: 10mM Phosphate Buffer, 150mM NaCl at pH 7.4 at room temperature; **B2**: 10mM Phosphate Buffer, 150mM NaCl at pH 7.4 at 0°C; **B3**: 0, 01 M Tris-HCl,150 mM NaCl pH8; **B4**: 50 mM Citrate buffer pH 5; **B5:** 0, 01 M Tris-HCl ,300 mM NaCl pH8; **B6**: 50 mM Citrate buffer, 300 mM NaCl pH 5). In the last wash, 100µl of each sample was collected in a half-area UV-Star 96-Well microplate, and the absorbance was read at 280nm. The last wash was performed when the absorbance values were lower than 0.005.

Then, 0.5ml of pure HSA (0.5 mg/mL) in different binding buffers was loaded in the column and 0.5ml of pure IgG (0.5 mg/mL) with the same method was added in other column. The protocol following is the same described in III.3.2.2 (Fig.13). Was made several washes with the bindings (5 cycles). In the end the elution buffer selected for each ligand A3A2 (10mM PBS w/ 50 % ethylene glycol pH 7.4) and for A6A5 (25 mM sodium phosphate, 150 mM sodium chloride, 30 mM sodium caprylate pH 6) were added (0.5 mL) in each column.

After, the total protein was quantified on the samples using the BCA assay kit. For this 25µl of the each sample was added with 200µl of the BCA reagent was added to each sample and incubated at 37ºC for 30 minutes. The absorbance of the samples was then measured in the microplate reader at 560nm, with a calibration curve using solutions with known protein concentration BSA in 10mM Phosphate Buffer 150mM NaCl at pH 7.4 from 0 to 200µg/ml with the values

After the screening, the columns were washed with the following solvents: 0.1M glycine-NaOH at pH 9.0 in 50% Ethileno glycol (v/v) (1×); 0.1M Glycine – NaOH at pH 11 (1×); distilled water (1×); 0.1M HCl (2×); distilled water (1×); 0.1M Glycine at pH 2.5 (1×); 0.2M NaOH in 50% isopropanol (v/v) (2×); distilled water (1×); 10mM Sodium Phosphate in 1M NaCl (1×); 0,1 M NaOH in 30% isopropanol (1×). Then, were stored at 4°C with 20% ethanol.

III.3.2.6 - Screening of the lead ligands in column format against plasma

This test was done in triplicate. After packing the columns (0.5g each column) with ligands-functionalized were washed with regeneration buffer (0.1M NaOH in 30% isopropanol)

followed by distilled water (3 cycles of washes, with the maximum volume of column). Then, ligands were equilibrated with the addition of the differents binding buffers (**B1**: 10mM Phosphate Buffer, 150mM NaCl at pH 7.4 at room temperature; **B2**: 10mM Phosphate Buffer, 150mM NaCl at pH 7.4 at 0°C; **B3**: 0, 01 M Tris-HCl,150 mM NaCl pH8; **B4**: 50 mM Citrate buffer pH 5; **B5:** 0, 01 M Tris-HCl ,300 mM NaCl pH8; **B6**: 50 mM Citrate buffer, 300 mM NaCl pH 5). In the last wash, 100µl of each sample was collected in a half-area UV-Star 96-Well microplate, and the absorbance was read at 280nm. The last wash was performed when the absorbance values were lower than 0.005.

The affinity ligands were screening against plasma, this test was done in column format. With a concentration of 0.7 mg/mL and dissolve in 50 mM sodium citrate, 300 mM NaCl pH 5 for the ligand A6A5 and 10mM Phosphate Buffer, 150mM NaCl pH 7.4 for the ligand A3A2.

After, the total protein was quantified on the samples using the BCA or the QuantiProTM BCA assay kit. For this 25µl or 150µL of the each sample was added with 200µl or 150µL of the BCA reagent was added to each sample and incubated at 37ºC for 30 minutes or 1h at 60ºC . The absorbance of the samples was then determined in the microplate reader at 560nm, with a calibration curve using solutions with known protein concentration BSA in 10mM Phosphate Buffer 150mM NaCl at pH 7.4 from 0 to 200µg/ml with

III.3.3 - Protein quantification methods

III.3.3.1 - SDS-PAGE preparation

In this work were used 12.5% acrylamine/bisacrylamine SDS-PAGE gels. The volumes of each solution used to prepare the gels are shown in Table 9. The first step was preparation of the running gel and the solution was transferred to the glass plates of the casting frame. In the end 100 µL of isoproponal was added to avoid bubbles, and the gel was polymerized for 30minutes.

Afterwards the isopropanol was removed and the stacking gel was added. The 5% acrylamide stacking gel was prepared and the mixture was added on top. The comb was inserted and the gel was polymerized for 15minutes. The electrophoresis buffer (0.25M Tris-Base, 1.92M glycine and 0.1% SDS pH8.3) was added to the tank and the polymerized gel was introduced in the running module. The samples prepared previously were applied in each well. The running conditions were set as 90V for 1:30 min.

Table 9 – Volumes necessary to prepare a 12.5% acrylamide gel for SDS-PAGE.

Stock solutions	Running gel 12.5% Acrylamine (µL)	Stacking gel 5% Acrylamine (µL)
Solution I (3M Tris Base pH 8.8)	750	-
Solution II (0.5M Tris Base pH 7)	-	450
Solution III (Acrylamine:Bisacrylamine 37.5:1)	2080	300
SDS 10%	50	18
dH_2O	2100	940
APS 10%	38	13.5
TEMED	2.5	2.0

III.3.3.2 - Sample preparation for SDS-PAGE analysis

The samples were normalized with the results of the BCA (1200ng). It was prepared 25 µL of each sample with sample buffer and it was boiled. 15µl were used to apply in the gel.

III.3.3.3 - Silver staining

The gel was transferred into the fixative enhancer solution (50mL methanol, 10mL acetic acid, 30mL distilled water and 10mL fixative solution from Bio-Rad) and incubated for 20 minutes with agitation. Afterwards the gel was rinsed twice in 200ml of distilled water for 10min.After this time, the staining solution (2.5mL silver complex, 2.5mL reduction solution, 2.5mL image solution, 25 mL accelerator solution from Bio-Rad kit and 17.5mL distilled water). In the end of the reaction the gel was incubated in 5% acetic acid solution to stop the reaction for 5 minutes. The gel was rinsed in distilled water.

III.3.4 - Partition equilibrium experiments

III.3.4.1 - Static partition equilibrium studies

These studies the tests were done in duplicate where the affinity ligand was the solid phase and mobile phase was HSA, target to which the affinity ligand is designed. The concentration of HSA was 0-3.5 mg/mL and the plate was incubate for 1h at 25°C. In the end was quantified the loadings and the flow-through with QuantiProTM BCA assay kit.

III.3.4.2 - Dinamic Binding Capacity

For determined Dynamic Binding Capacity was used Tricorn™ empty high performance columns (GE Healthcare, Uppsala, Sweden), with 5.4mL of affinity ligand A6A5 (3g), using it AKTA

pure (GE Healthcare).Was used two different concentration 0.5 mg/mL and 10 mg/mL to saturate the resin.

III.4 - Results and discussion

Human serum albumin and immunoglobulins, highly abundant serum proteins, are one of the major obstacles in proteomics analysis of serum or plasma samples. Proteins present in serum have a dynamic range of concentration so it necessary remove proteins that mask the signal to allow detection and analysis of less abundant serum proteins. So, depletion of the most abundant proteins in human serum will significantly increase the sensitivity in analysis of the remaining proteins.

In addition, the serum albumin is used for many medical applications and are produced more than 500 tons of HSA and more than 40 tons of intravenous immunoglobulins, once HSA consumption in the developed world is 200-400 kg per million population [1].

For this purpose, was develop two libraries, one based on Triazine reaction and other on Ugi reaction (Annex I). Three ligands are selected A3A2 and A6A5 from Triazine library, and C7A3 from Ugi reaction. This ligands show selectivity for HSA in comparison with polyclonal IgG.

In this work, after selection of the ligands more specific for HSA, we proceeded to optimization of conditions of the binding and elution to obtain higher purity and high yield from plasma samples. For that, several screenings against pure HSA, pure IgG and plasma were accomplished. In the end, the best ligand candidate was explored in terms of dynamic binding capacity and studies of static partition equilibrium was accomplished.

III.4.1 - Enrichment Factor

The ligands were selected from libraries made by master student Aline Viecinski in Biomolecular Engineering Lab (Annex I), and the first study was certificated the veracity of the results that was obtain, for that was realized the enrichment factor (Equation 1). This result was made by tested the ligands against HSA and IgG. For that was added HSA or IgG in concentration of 0.25 mg/mL dissolved in 10mM Sodium Phosphate, 150 Mm NaCl pH 7.4 (binding buffer used in previous work) to the binders and the results was determined by the following equation.

$$Enrichment\ Factor = \frac{\%\ HSA\ bound}{\%\ IgG\ bound} \qquad \text{Equation 1}$$

The results obtain for the QuantiProTM BCA assay, this assay gives a linear response from 0.5 to 200µg/mL, and the BSA gives a linear response a higher concentrations of protein (200-1000µg/mL). This techniques the quantification of the total protein are based on the formation of a Cu^{2+} protein complex under alkaline conditions. In proteins, cysteine, tryptophan, tyrosine and peptide bounds are able to reduce Cu^{2+} to $Cu^{1+,}$ producing a purple-blue complex which absorbs at 562 nm. The amount of reduction is proportional to protein.

The percentage of HSA bound and IgG bound are determinate by the following equations:

$$Amount\ of\ protein\ bound\ (ug) = Amount\ of\ protein\ loaded\ (ug) - Amount\ of\ protein\ washed\ (ug)$$

Equation 2

$$\%Bound = \frac{Amount\ of\ protein\ bound\ (\mu g)}{Amount\ of\ protein\ loaded\ (\mu g)} \times 100$$

Equation 3

The results shows that for Triazine ligands A3A2 and A6A5 the percentage HSA bound, 99 and 100% respectively, are superior then percentage IgG bound for the same ligands, 59 and 58% respectively (Fig.14 A). Verifying an enrichment factor superior to 1 that culminates with a selectivity the ligands A3A2 and A6A5 for HSA (Fig.14 B). In case of Ugi ligand C7A3 the difference was lesser than the other ligands, 45 % for HSA bound and 36 % for IgG bound, but was possible verify the preference for binding albumin rather than IgG.

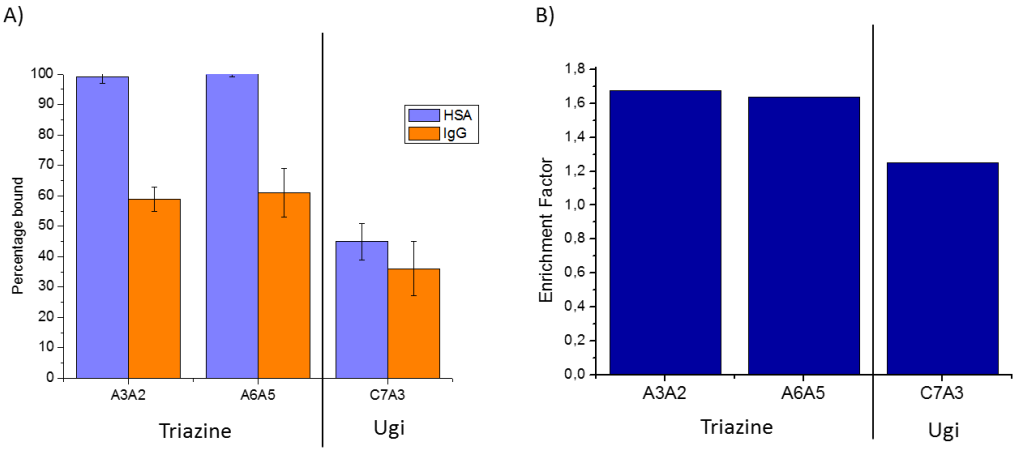


Figure 14- Enrichment Factor analyses. (A) Screening results of lead affinity ligands with 10Mm Phosphate Buffer at pH 7.4 against HSA and IgG. (B) Enrichment Factor of each selected ligand.

III.4.2 - Analyses of structure of ligands

For optimized the conditions of binding and elution was analyze the structure of ligands A3A2, A6A5 and C7A3. For that purpose, the structures were characterized in terms of type of interactions that can happen and was calculated the pKa values of each function group by the software MarvinSketch.

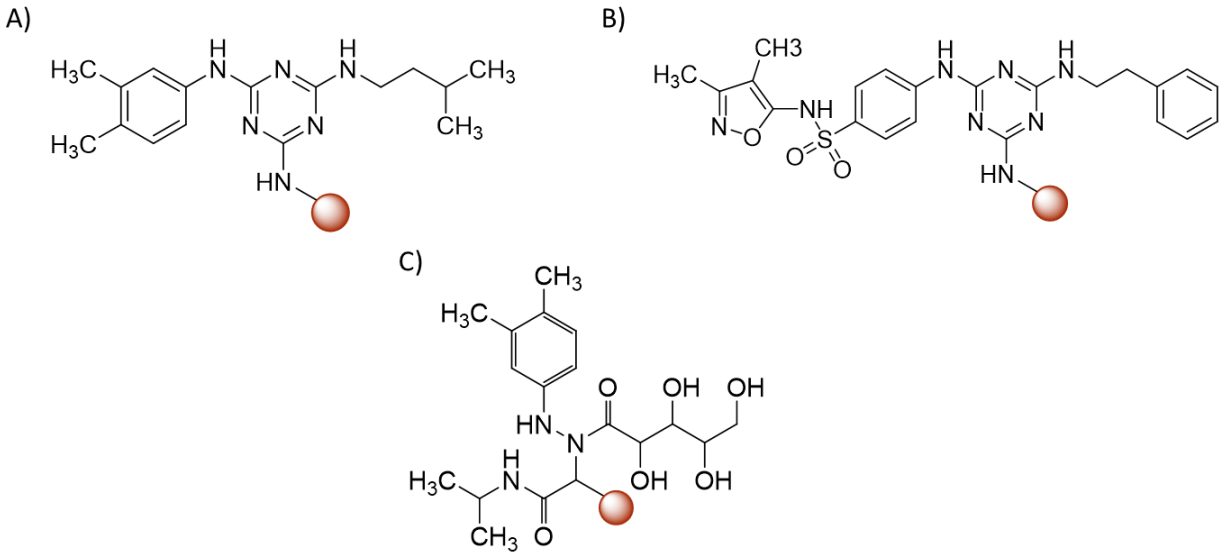


Figure 15- Representation the structure of the affinity ligands. (A) Triazine ligand A3A2 (B) affinity ligand A6A5 (C) Ugi ligand C7A3.

Triazine and Ugi ligands present aromatic compounds that promote hydrophobic interactions. This interaction contributes for column selectivity against HSA, but also can promote nonspecific interactions with the other proteins to whom ligands are not designed.

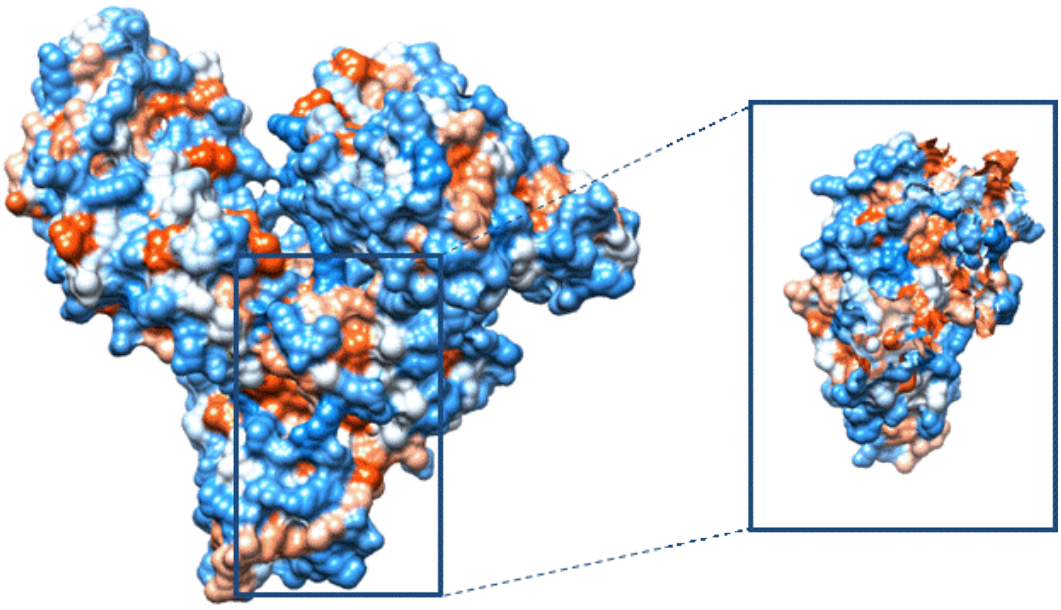


Figure 16- Hydrophobicity Surface analysis from a crystallography structure of HSA, with a zoom in the domain II. Blue represent the most hydrophilic regions and red the most hydrophobic regions. Image obtain from Chimera1.10.1 software. PDB code 109X

According with Figure 15 the structure of ligands the main interactions existing are the hydrophobic interactions due to the aromatic groups, the cis-diol corresponding to the methyl group and hydrogen bounds consistent with the hydroxyl and amine function groups.

Viewing the domain II of HSA (Figure 16), where the ligands were designed to interact is possible to see that the active site was mainly hydrophobic making possible the hydrophobic interaction with the affinity ligands.

Taking into account these observations, we proceeded to optimization of the elution and binding buffers with objective of purification of HSA from human plasma.

III.4.3 - Choose of the elution buffers

In first step several elution buffers already known as elution buffer for HSA in commercial kits used for purification. Eight different buffers were prepared. **E1**: 0.1 M glycine pH 3; **E2**: 25 mM sodium phosphate, 150 mM NaCl, 30 mM sodium caprylate pH 6; **E3**: 10mM PBS w/ 1M NaCl pH 7.4; **E4**: 50 mM sodium phosphate, 1.5 M KCl pH 6; **E5**: 0.1 M Glycine-NaOH pH 10; **E6**: 150 mM ammonium acetate, 10 mM sodium caprylate pH 6; **E7**: 10mM PBS w/ 50 % ethylene glycol pH 7.4; **E8**: 10mM PBS w/ 2M $MgCl_2$ pH 7.4

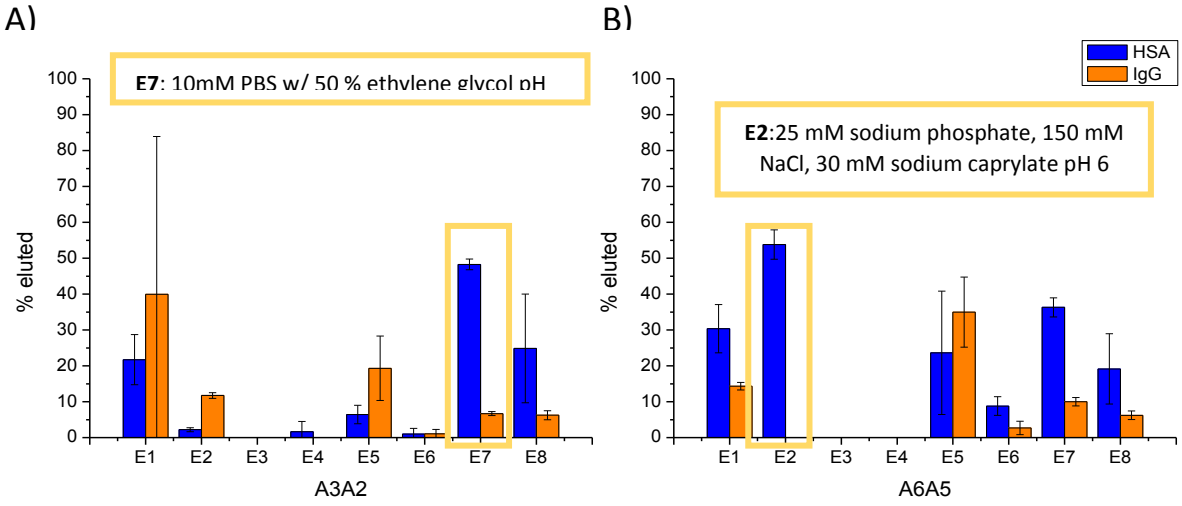


Figure 17- Screening results of the lead Triazine ligands, with different elution conditions (E1-E8). For the ligand A3A2 was selected the elution E7: 10mM PBSW/ 50% ethylene glycol pH 7.4 (A) and for the ligand A6A5 was selected the elution E2: 25 mM sodium phosphate, 150 mM NaCl, 30 mM sodium caprylate pH 6 (B)

Each elution with different properties, different pH to change the protonation state of the ligands, different concentration of salts, different organic compounds.

The results were obtained with the constant binding buffer 10mM Sodium Phosphate, 150mM NaCl pH 7.4 (Fig.17). The results from Ugi ligand C7A3 was not showed because with this elution conditions, no proteins was eluted.

With this results was possible to see that the ligand A3A2 as A6A5 elute more HSA than IgG except with the elution that have the glycine with pH3 and pH 10, in this case the percentage of eluted IgG was superior than HSA. In case of E3 and E4 nothing was eluted not HSA or IgG except one little percentage with the E4, in A3A2, in this two elution buffers the concentration of salt was variable, so concludes that concentration of salt not is one important factor for elution. Being possible to watch with E8, an elution with magnesium chloride, was the unique buffer that the only variable was the concentration of salt (2M) that show positive results (HSA was eluted 20 to 30 percent and IgG 0 to 20 percent). This results is consistent with the increased of strength of hydrophobic interaction with the increase of salt concentration [50], however shows that a 2 M exist elution of the proteins.

At different pH, there is a change in the protonation state of ligands and effect the strength of ligation with proteins allowing their elution. For this purpose was calculated the pKa values of each function group by the software MarvinSketch (Fig.18).

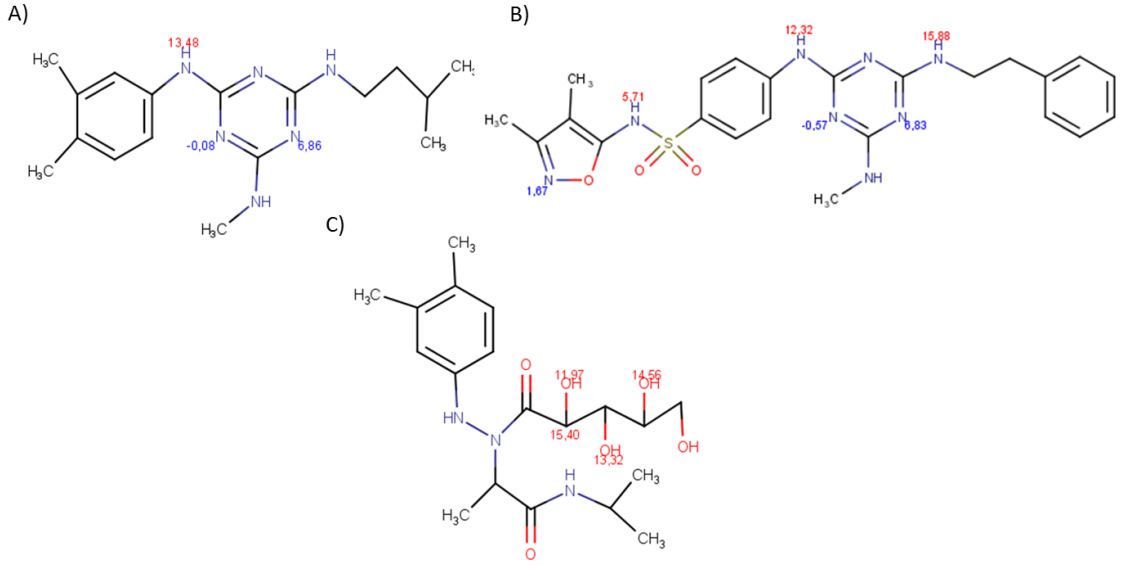


Figure 18- pKa values of lead ligands. Values were determined using the pKa Plugin from MarvinSketch (ChemAxon). The methyl group was included in the structures of the ligands in place of the agarose bed. (A) A3A2; (B) A6A5;(C) C7A3.

The elution buffers have a variation of pH from 3 with glycine buffer until pH 10 also glycine, but the range pH 6 to 7.4 was the best observed. In this range is possible to see with the results that in ligand A3A2 the pH 7.4 with ethylene glycol was the best, proving in Figure 17 that

these pH the only deprotonated this in aromatics Figure 18 (A), while for ligand A6A5 the best elution was a pH 6 and in this pH the aromatics from triazine and the amine A6 (Sulfixazole) are on the deprotonated state (Fig 18 (B)). For the Ugi affinity ligand C7A3 was possible see that the pKa from the functional groups are in range of pH 11-15 and this may be a reason why there is not elution of proteins because all of the elution conditions not reach so high pHs (Figure 18 (C)).

In the end, the best condition for elution was **E7**: 10mM PBS w/ 50 % ethylene glycol pH 7.4 for A3A2 and **E2**: 25 mM sodium phosphate, 150 mM NaCl, 30 mM sodium caprylate pH 6 for A6A5. In buffer **E7** with 50 % ethylene glycol, was used an organic compound, an alcohol with two group hydroxyl (-OH) that possibly interferes with the hydrophobic interaction of A3A2 to the HSA and was used **E2** with sodium caprylate, an eight-carbon saturated fatty acid that can compete for active site that A6A5 interacts and in this case the HSA shows more preferences for these compound so exist elution from HSA from plate.

For the next step of the work, it was decided not continue with Ugi affinity ligand C7A3 because besides having a small percentage of bound HSA not elutes to any elution condition studied and in the next studies the elution buffer with the best result obtained from screening (Figure 17) **E7**: 10mM PBS w/ 50 % ethylene glycol pH 7.4 for A3A2 and **E2**: 25 mM sodium phosphate, 150 mM NaCl, 30 mM sodium caprylate pH 6 for A6A5 was used.

III.4.4 - Optimization of the Binding Buffer

For previous work the binding buffer used was 10mM Sodium phosphate, 150mM NaCl pH 7.4 with this buffer the percentage of HSA bound for the two ligands was approximate 100 percent but the percentage of IgG was high therefore the purpose was to optimize the binding buffer to increasse specificity for HSA than IgG, having a decrease of IgG bound.

For this phase was selected four different conditions of binding **B1**: 10mM Sodium phosphate, 150mM NaCl pH 7.4 at room temperature; **B2**: 10mM Sodium phosphate, 150mM NaCl pH 7.4 at 0°C; **B3:** 0.01M Tris-HCl,150mM NaCl pH 8; **B4**: 50mM citrate buffer pH 5 selected from kits that already exist modifying them for the purpose.

According with the results for the ligand A3A2 (Figure 19) was possible see that the results from plate and the results obtained with the reading the absorbance at 280 nm are similar in elution (Figura 19 (C, D)), what not what happening with the results obtain with BCA, possible due to dialyze step. In the other hand, the percentage of protein bound (Figure 19 (A,B)) was similar in test in plates and column it is therefore dialyses considered the factor that

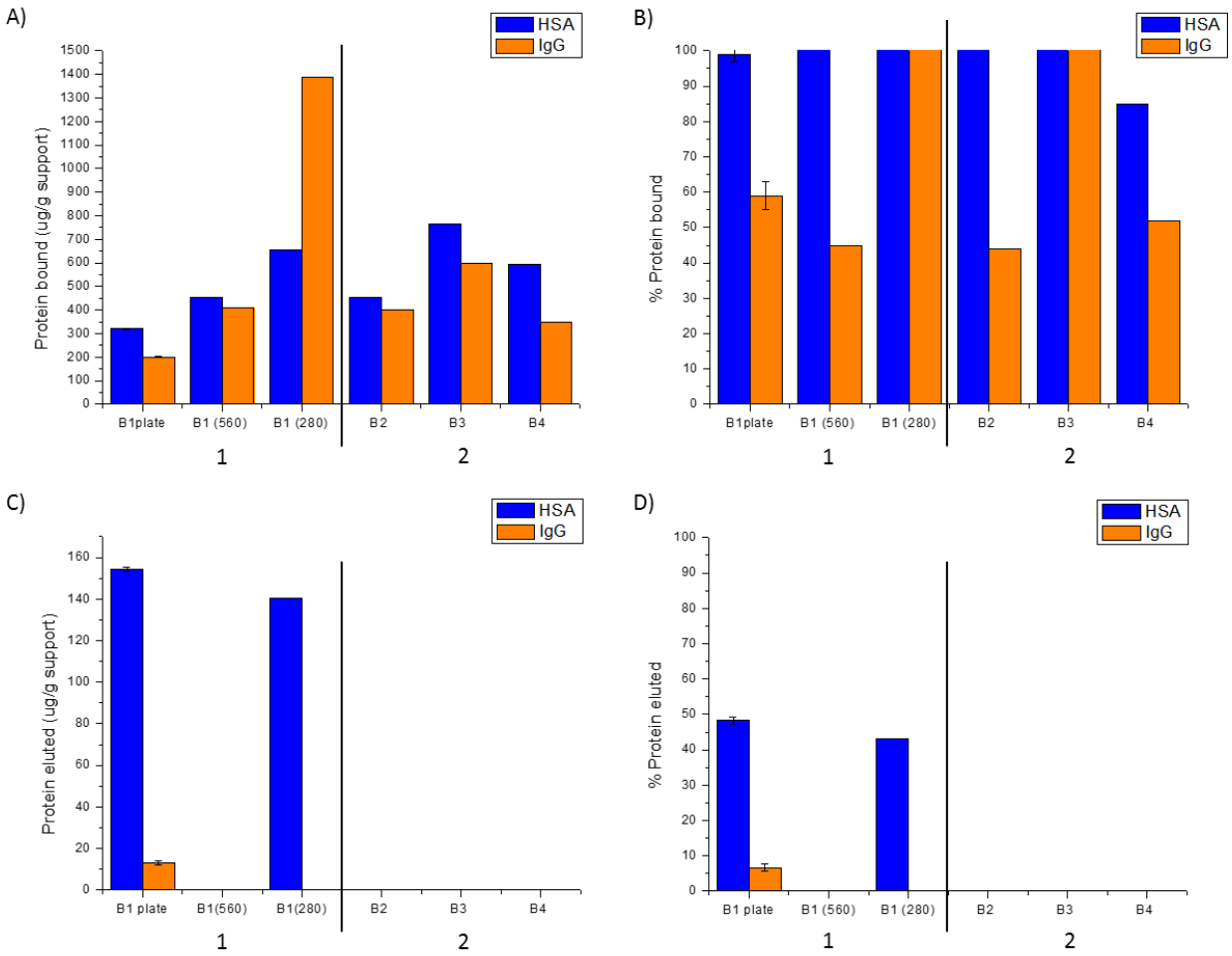


Figure 19- Screening results of Triazine ligand A3A2, with different binding conditions (B1-B4) and elution E7 (10mM PBS w/ 50 % ethylene glycol pH 7.4). (A) The binding results of protein bound (ug/g support). (B) The percentage of protein bound. (C) The elution results of protein eluted (ug/g support). (D) The percentage of protein eluted. (1) Comparison of results obtain in plate (triplicate quantified by microBCA) and in column (BCA assay and 280nm). (2) Remaining binding conditions in column (BCA).

influenced the results of elution step. In case of ligand A3A2 the binding buffer wasn't optimized and for the future work in the screening with plasma was used binding buffer **B1**: 10mM Sodium phosphate, 150mM NaCl pH 7.4 at room temperature and elution buffer **E7**: 10mM PBS w/ 50 % ethylene glycol pH 7.4.

For the ligand A6A5 the same binding buffers was used and the results are show in Figure 20. According with the results was possible observed that in terms of comparison between the screening in plate and columns that the percentages was similar only existing difference in the percentage of IgG eluted through the column. With this ligand only HSA was eluted from column with the binding buffers (Figure 20 (C, D). The only difference between B1 and B2 was the temperature of the buffers, room temperature and 0ºC respectively, and the objective

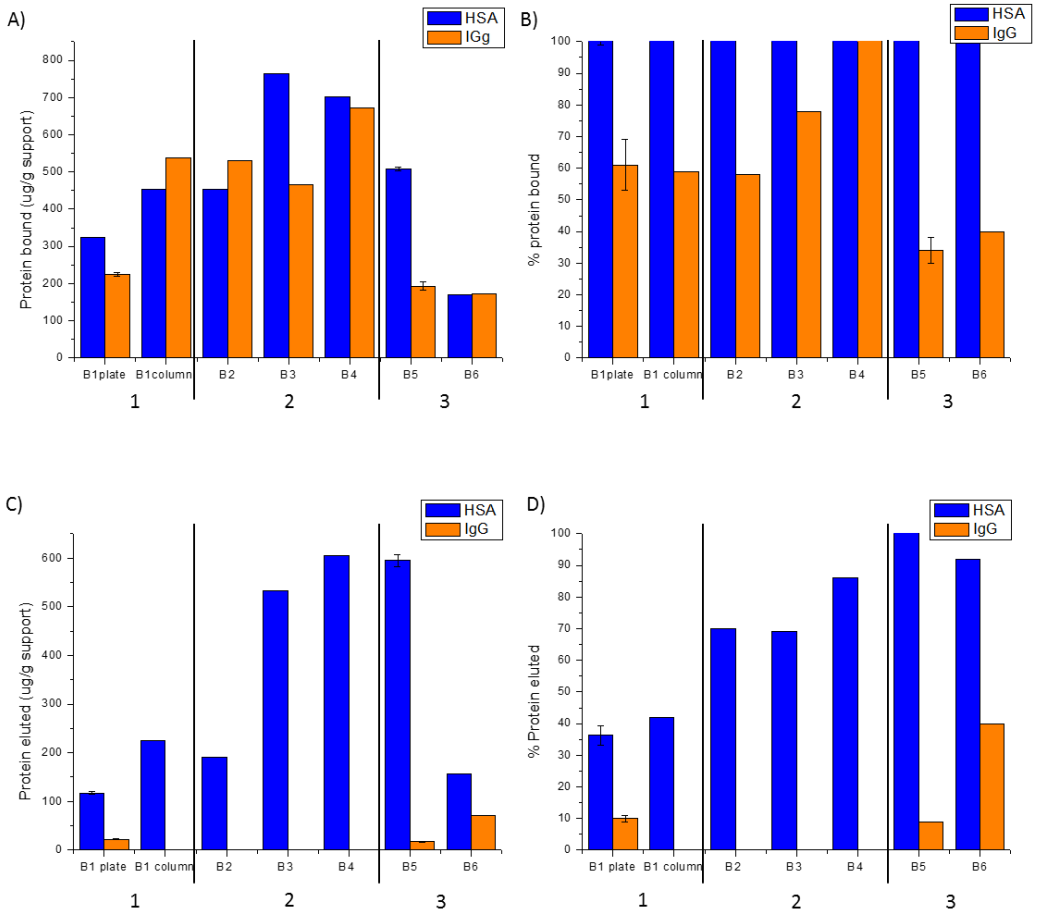


Figure 20- Screening results of Triazine ligand A6A5, with different binding conditions (B1-B4) and elution E2 (25 mM sodium phosphate, 150 mM NaCl, 30 mM sodium caprylate pH 6). (A) The binding results of protein bound (ug/g support). (B) The percentage of protein bound. (C) The elution results of protein eluted (ug/g support). (D) The percentage of protein eluted. (1) Comparison of results obtain in plate (triplicate quantified by microBCA) and in column (BCA assay.(2) Remaining binding conditions in column (BCA). (3) Optimization of binding buffers B3 and B4 to B5 (duplicate) and B6, respectively.

with this work, was the decrease of percentage the IgG bound, once the increase of temperature increase the hydrophobic interaction [51], however with the results was possible observe that the percentage of IgG not decreases with the decrease of temperature observing that the IgG possibly interact with the A6A5 through hydrophobic interactions.

Besides used the PBS as buffer was used Tris-HCL (B3) with salt (150mM NaCl) that as noted in Figure 17, the elution with concentration of salt nothing elutes considering that the concentration of salt promotes the hydrophobic interactions [50] between the ligand and the HSA. With 0.1 M Tris-HCl, 150mM NaCl pH 8, the ligand A6A5 was deprotonate in functional group amine A6 (Sulfixazole) and was possible to see that the increase of salt increases the percentage of IgG bound comparatively with the percentage in B1 and B2, concluded that IgG possibly interacts with A6A5 through hydrophobic interactions as already suspected with decreasing temperature in buffer B2.

In this work was used also the B4 with citrate and in this case the percentage of IgG was superior than when was used the buffer B3. The buffer used have the pH close of pKa of amine A6 may be a factor that influences the interaction of IgG and HSA with A6A5. The citrate as a conjugate base of a weak acid is used in the formation of buffers. This compound have in your structure free carboxylic acids that are studied as a good coupling directly with the agarose beds [12], in this case the citrate conjugate with the pH helps in interaction between IgG and HSA to the A6A5 so exists 100 percent of HSA and IgG bound to the agarose.

The next stage was to optimize the bindings picking up buffers with the best percentage of HSA and IgG bound. Once the ligands did not show more specificity for HSA than IgG, the binding B3 and B4 were optimized, and how the previous experience shows that the with increase of salt exist a increase the hydrophobic interaction, two new buffers were elaborated **B5**: 0. 01M Tris-HCl, 300mM NaCl pH 8 and **B6:** 50mM citrate buffer, 300mM NaCl pH 5 (Figure 20 section 3).

The idea was simply to increase the hydrophobic interactions for the B5 in comparison with the B3 was possible see that the percentage of IgG bound was inferior but the percentage of HSA eluted with the elution buffer previous choose was superior. In contrast, the B6 the percentage of HSA keeps and the percentage of IgG decreases and the percentage eluted of IgG increases comparatively with B4.

In the end of this phase we selected **B5** and **B6**, both buffers show a decrease of specificity for IgG comparatively with the buffers natives and show a percentage of elution the HSA approximately 100 percent.

III.4.5 - Optimization of the Elution Buffer for ligand A6A5

After optimizing the binding buffer increasing the concentration of salt thereby increasing the interactions hydrophobic, the elution buffer was also optimized. In this case the buffer previous applied was modified with the opposite point of view of optimization of binding buffers, if the increase of concentration decreases the percentage of IgG bound the opposite helps the IgG stay bound and supposedly HSA is eluted. The first elution was **E2**: 25mM sodium phosphate, 150mM NaCl, 30mM sodium caprylate pH 6 and in this stage was tested two different buffers **E9**: 25 mM sodium phosphate and **E10**: 25 mM sodium phosphate, 30 mM sodium caprylate pH 6.

Table 10 - Screening results of optimization of elution buffers used for the ligand A6A5 with the two binding buffers (B5 and B6). E9: 25mM sodium phosphate; E10: 25 mM sodium phosphate, 30 Mm sodium caprylate pH6; B5: 0. 01M Tris-HCl, 300mM NaCl pH 8; B6: 50mM citrate buffer, 300mM NaCl pH 5

	HSA bound	IgG bound	HSA E9	HSA E10
			IgG E9	IgG E10
Buffer B5	250 ± 5 µg/g suport 97 ± 2%	115 ± 2 µg/g suport 68 ± 1%	0µg / 0%	180µg/74 %
			2µg/ 2%	2µg/2%
Buffer B6	188 ± 0 µg/g suport 99 ± 0%	126 ± 5 µg/g suport 96 ± 4%	0µg/0 %	200µg/ 100%
			0µg/ 0%	0µg /0 %

The percentage of IgG increase in percentage of protein bound to the ligand, namely with buffer B6, this event is due to the fact that the BCA assay has less sensitivity that QuantiProTM BCA assay has.

The difference between the E9 and E10 was in the presence of sodium caprylate in this case was possible observing the importance of caprylate in the elution step. Table 10 summarizes the quantification realized with BCA assay, and show that caprylate have the principal rule in elution of HSA. In this step the E10 was the better with the buffer B6, although bind 100 percent of HSA and IgG, exists only HSA elution. How was referred the elution without NaCl decreases the hydrophobic interactions, there elution of HSA while IgG remains on the resin.

For the screening with plasma with the affinity ligand A6A5 was used the optimized conditions **B6**: 50mM citrate buffer, 300mM NaCl pH 5 and **E10**: 25 mM sodium phosphate, 30 mM sodium caprylate pH 6, and for the ligand A3A2 was used buffer **B1**: 10mM Sodium phosphate, 150mM NaCl pH 7.4 at room temperature and elution buffer **E7**: 10mM PBS w/ 50 % ethylene glycol pH 7.4.

III.4.6 - Screening against plasma

After optimizing the binding and elution buffers the ligands were tested for binding to human proteins plasma with a concentration of 0.7 mg/mL. In the end was used the 12.5 %acrylamine/bisacrylamine SDS-PAGE gels, to see which proteins were eluting from column.

Table 11 - Screening results of the A3A2 and A6A5 ligands with Human Plasma in the best binding and elution conditions (n=2).

A6A5		
	% Total protein	Mass total protein (ug/g support)
Buffer B6	87 ± 4	316 ± 16
Buffer E10	47 ± 2	147 ± 4

A3A2		
	% Total protein	Mass total protein (ug/g support)
Buffer B1	100 ± 0	399 ± 0
Buffer E7	55 ± 1	220 ± 1

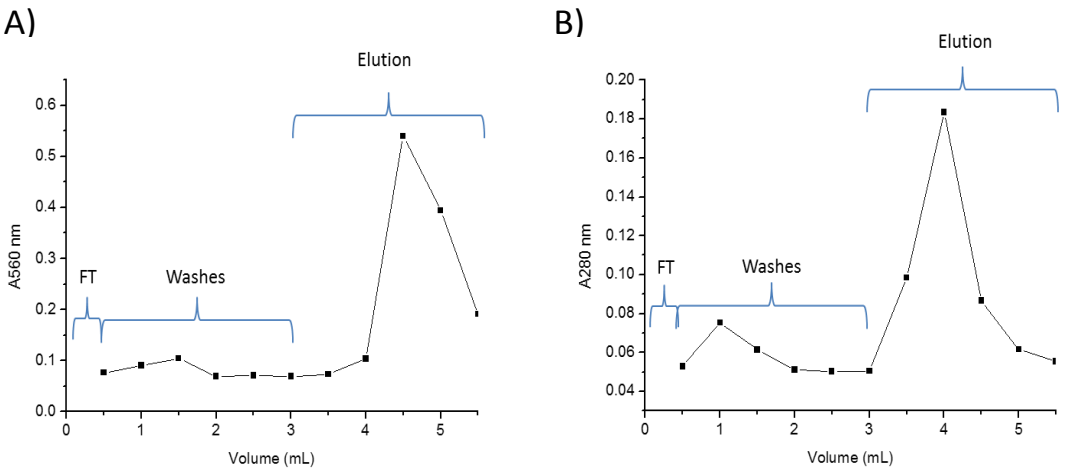


Figure 21-Chromatogram obtained for the screening results of the affinity ligands A6A5 (A) and A3A2 (B) with Human Plasma in the best binding and elution conditions. The screenings were performed on-column, in duplicate.

With the quantification by BCA assay it was possible to quantifing the total protein bound and eluted from column for ligand A6A5 (Table 8 and Figure 15 A), the quantification of ligand A3A2 was made by reading the absorbance at 280 because the elution buffer interferes with the BCA assay.

How the quantification is the quantification of total protein was necessary, confirm the results with a gel SDS- PAGE for the both ligands.

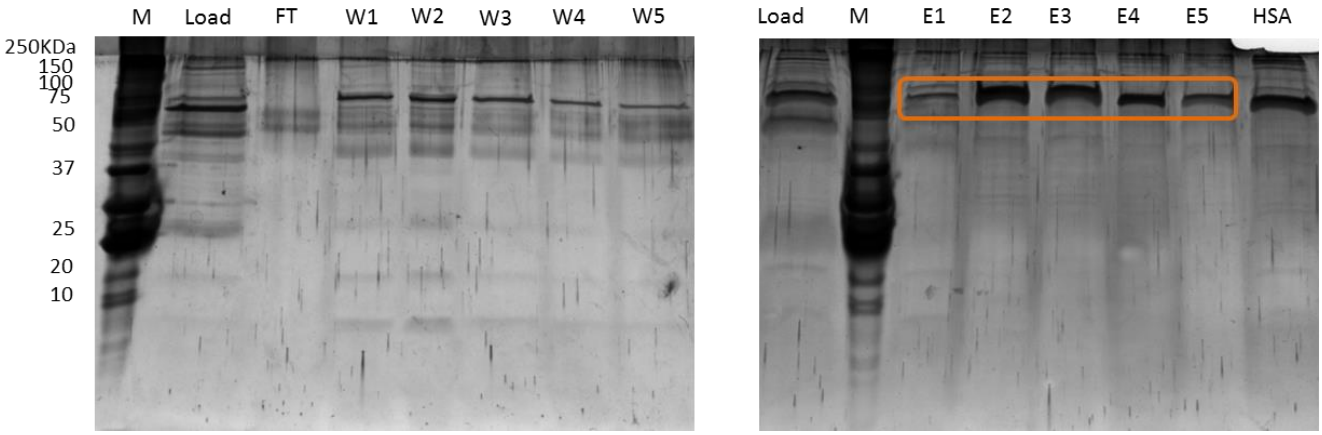


Figure 22-SDS-PAGE gel obtained for Human Serum Albumin purification from human plasma with A6A5 affinity ligand. The molecular marker, loading, pure HSA, flow-through, washes and elution fractions correspond to M, L,HSA, FT, W and E, respectively. The present gels are 12.5% acrylamide and stained by silver staining. Orange box represent the HSA band (66kDa).

According to the gels ligand A6A5 (Figure 22) was possible identify the band of HSA (orange) with 66kDa, verifying that the only protein existent in elution step was HSA (having in comparison the HSA pure also placed in the gel). However, the gels of ligand A3A2 (Figure 23) shows that several proteins are present in elution step (is possible see the band of IgG in blue).

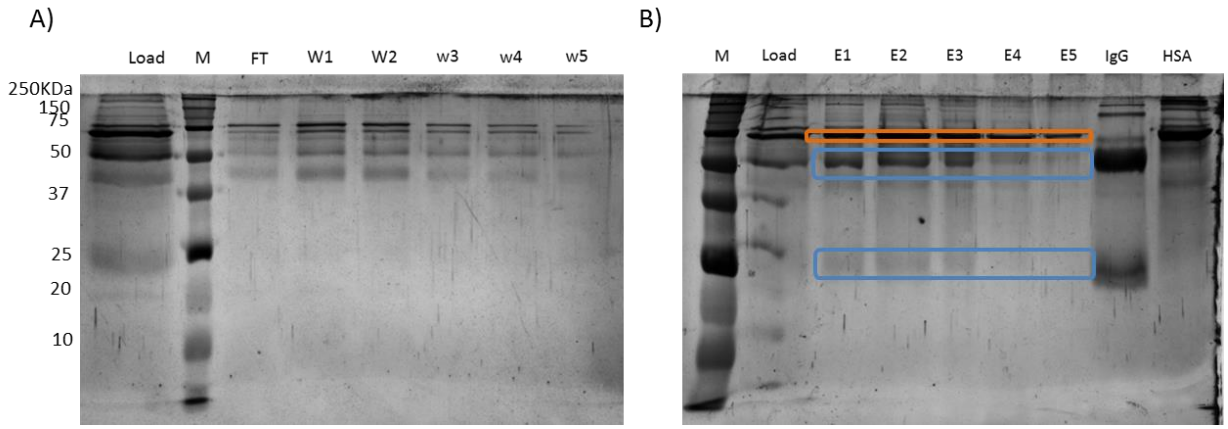


Figure 23- SDS-PAGE gel obtained for Human Serum Albumin purification from human plasma with A3A2 affinity ligand. The molecular marker, loading, pure HSA, flow-through, washes and elution fractions correspond to M, L,HSA, FT, W and E, respectively. The present gels are 12.5% acrylamide and stained by silver stainin g.Orange box are presented the HSA band (66kDa) and in blue the IgG bands (50kDa and 25kDa)

The purity of the eluted fraction has been calculated by the ImageJ. software. The results regarding this system are summarized in Table 12.

Table 12 - Percentage of HSA purity, after purification with affinity ligands A3A2 and A6A5 calculated from ImageJ software.

Ligand	Purity (%)
A6A5	93
A3A2	37

With 93% of purity of HSA in A6A5 comparatively with 37% in A3A2, we can conclude that the affinity ligand A6A5 is the best candidate for the purpose of the work. The optimization of binding and elution steps for ligand A6A5 successful.

III.4.7 - Static partition equilibrium studies for the determination of binding constants

The affinity binding constant (Ka), that describes the strength of the interaction between an affinity ligand and a protein, in this case HSA (Figure 24) and the maximum binding capacity of the support (Q_{max}), was obtained from the application of a Langmuir model to data from partition equilibrium studies.

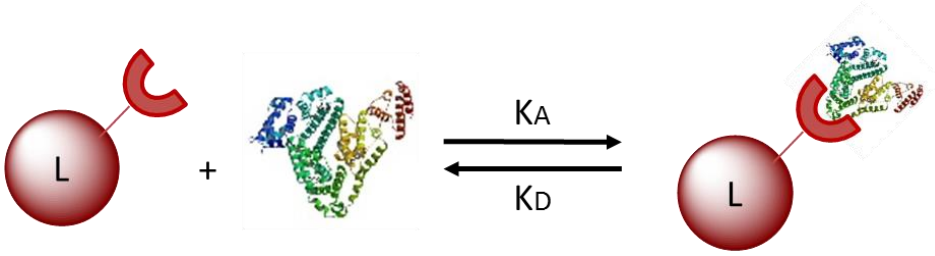


Figure 24-Schematic representation of the interaction between an immobilized affinity ligand and a target protein.

The Langmuir isotherm was employed in affinity ligand A6A56 and is described by Equation 4

$$q = \frac{Qmax \times Ka \times C}{1 + Ka \times C} \qquad \text{Equation 4}$$

Langmuir adsorption isotherm. Where q is the binding capacity (mg HSA bound/g support); Qmax is the maximum binding capacity (mg bound/g support); Ceq is the concentration of the protein in solution at the equilibrium (mg/mL); and Ka is the association equilibrium constant (mL/mg).

A) B)

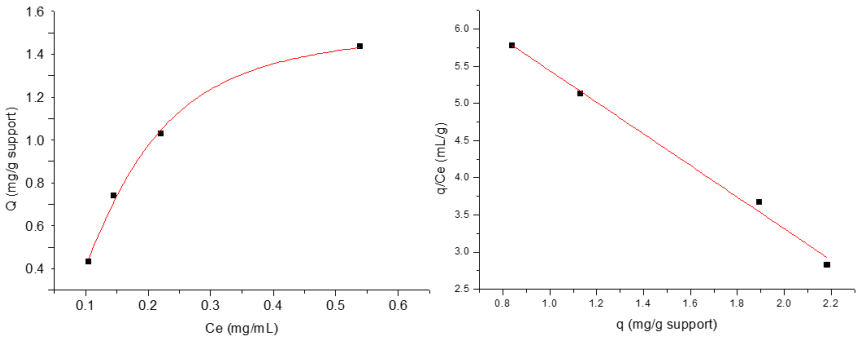


Figure 25-Binding isotherms for the affinity ligand A6A5.The experimental results have been fitted to the Langmuir model (A) and Scatchard plot (B).

Beyond the Langmuir isotherm was used the Scatchard plot to obtain the binding constants Ka (M^{-1}) and Qmax (mg protein bound/g support), this being described by the Equation 5. The resulting constants are summarized in Table 13.

$$\frac{q}{Ce} = KaQmax - Kaq \qquad \text{Equation 5}$$

Table 13 - Binding constants Ka (M^{-1}) and Qmax (mg protein bound/g of support) after fitting with Langmuir model and Scatchard plot. R^2 is the correlation factor.

A6A5	Qmax (mg/g support)	Ka (M^{-1})	R^2
Langmuir	1.63 ± 0.07	3.76E+06	0.99
Scatchard	3.14	1.4E+05	0.99

The Ka values obtained was in the range considered for suitable purification methods where Ka 10^3-$10^9 M^{-1}$[52] .In terms the results of Qmax was obtains 1.63 mg/ g support for Langmuir and 3.1 for Scatchard plot, values superiors obtain previous for affinity ligands immobilized on agarose [53].

III.4.8 - Dynamic Binding Capacity

The Dynamic Binding Capacity was determined with use a Tricorn™ empty high performance columns (GE Healthcare, Uppsala, Sweden). The 3g of affinity ligand A6A5 was tested against HSA, target protein that resin was designed for. Was tested two different concentrations 0.5 mg/mL and 10 mg/mL of HSA and the column never saturates. In conclusion, the dynamic binding capacity of the affinity ligand is bigger than 10mg/mL.

III.5 - Conclusion

This Chapter was a continuation of a previous work performed at the Biomolecular Engineering Lab by master student Aline Viecinski. The first part of the work was developed two affinity ligand libraries, based on Triazine and Ugi reaction. The ligands were designed to mimic the interaction between Human Serum Albumin with natural protein PAB. From the screening against HSA and IgG with these libraries resulted there lead ligands, two Triazine affinity ligand A3A2 and A6A5, and one Ugi ligand A6A5. These ligands show more specificity for HSA than for IgG.

The main goal of this work was to optimize the conditions to obtain a better affinity absorbent for HSA purification. Firstly, was tested different elution conditions with purpose to find a buffer that selectively unbind HSA. In this case was selected the elution E7 (10mM PBS w/ 50 % ethylene glycol pH 7.4) for A3A2 and E2 (25 mM sodium phosphate, 150 mM NaCl, 30 mM sodium caprylate pH 6) for A6A5.For the next phase of the studies only the Triazine ligand were tested.

Then, Triazine ligands proceeded for the optimization of binding towards HSA, for the ligand A3A2 the best condition was B1 (10mM Sodium phosphate, 150mM NaCl pH 7.4) and for the A6A5 was B6 (50mM citrate buffer, 300mM NaCl pH 5), used for increase the hydrophobic interactions. In the end, the elution conditions for the ligand A6A5 were optimized in order to decrease the hydrophobic interactions allowing the elution of HSA.

The tests with plasma were performed with the chosen buffers for each ligand and results were confirmed with SDS-PAGE. For the ligand A6A5 was obtain high HSA purity (93%) and for the ligand A3A2 was obtain 37% of HSA purity, which is low for the affinity chromatography ligands. To overcome this problem, an IgG depletion or an optimization of the buffers is needed.

The experimental data of partition equilibrium studies from the ligand A6A5 was fitted according to Langmuir isotherm. The affinity constant (Ka), are in the same range (3.76E+06) that was considered for purification methods and maximum binding capacity (Qmax), are superior to the ones found in literature.

In terms of Dynamic Binding Capacity the affinity ligand A6A5 didn’t saturate the resin resulting in DBC superior to 10mg/mL.

Chapter 4.
PURIFICATION OF HSA WITH BIOLOGICAL AFFINITY LIGANDS

The aim of this Chapter was to confirm by ELISA assays the affinity and selectivity of 3 protein sequences previously engineered and evolved to bind to HSA and IgG.

IV.1 - Introduction

In 2002, over 155 approved pharmaceuticals and vaccines had been developed by biopharmaceutical companies. Nowadays, more than 200 approved peptide and protein pharmaceuticals are on the FDA list. Human insulin, albumin, human growth hormone (HGH), Factor VIII are some of the recombinant protein pharmaceuticals produced. In 1993, over 50% of the industrial enzyme market was provide by recombinant processes and sales were 140 million dollars [54].

Affinity chromatography is the most well-established platform used method for protein purification because of the high yields of recovered protein and purity over 90% [55][56]. In this chromatography, selectivity towards a specific target protein is introduced through the chemical functionalization of the solid support with desired affinity ligands, which can be divided into three main categories: biological, structural and synthetic [57].

Biological ligands are biomolecules obtained from natural sources and from *in vitro* selection techniques. They have high selectivity and affinity for the target, but also have high costs of production and purification [55]. Novel biological affinity ligands can be obtained through in vitro selection techniques such as phage, ribosome or yeast display [58].For the technique phage display already exist ligands in drug delivery field and used in affinity purification (Table14) The existing biological affinity ligands include immunoglobulins against a target protein, bacterial immunoglobulin-binding domains such as proteins A, G and L, and natural lectins targeting glycoproteins [55].

Protein domains are natural modulators of biological function through ligand binding. These domains may be engineered in order to produce altered activities and novel affinity reagents. Engineering efforts focus upon modification or randomization of the loops in globular structures [58].

WW domains are small modules composed of approximately 40 amino acids in length and fold into a three-stranded, antiparallel β sheet with two ligand-binding grooves [59]. The name refers to two signature tryptophan (W) residues that are spaced 20-22 amino acids apart and play an important role in its structure and function [60], These domains comprise a family of protein-protein interaction modules that are found in many eukaryotes and are present in approximately 50 human proteins [61].

It is one of the smallest protein module that folds as a monomer without disulfide bridges or cofactors. WW domains bind proteins containing short linear peptide motifs that are proline-rich or contain at least one proline. WW domain initially was considered a “cytoplasmic

module", however the proteins containing WW domains have also been localized in the cell nucleus [60]. The WW domains bind a variety of peptide ligands including motifs with core proline-rich sequences, such as PPXY (amino acid single-letter code; X is any amino acid) (PY), PPLP, as well as proline/arginine-containing (PR) sequences and phosphorylated serine/threonine-proline sites [p(S/T)P] [61]. WW domain because the signaling complexes it mediates have been implicated directly or indirectly in several human diseases including Liddle's syndrome of hypertension, muscular dystrophy, Alzheimer's and Huntington's diseases, and, more recently, cancer [62][60]. Thereby, the WW fold may constitute a template into which binding sites from unrelated proteins may be used as a strategy to develop proteins with novel properties [63]

Table 14 - Comparison of the different HSA ligands

Ligand	Type	Target	Evolution	Aplication
ABD	Small protein domain based on Streptococcal protein G	Biospecificity anti-TNF-α and HSA	Phage display	Drug delivery
		HSA	Phage display	Drug delivery
		HSA	Phage display	Affinity purification
		Biospecificity Z domain of SpA and HSA	Phage display	Affinity purification
VHH domain	Derived from an Ig-fragment	Biospecificity anti-TNF-α and HSA	Phage display	Drug delivery
Affibody	Z domain derived from B domain of protein A	HSA	Phage display	Affinity Purification/ Depletion/ ELISA/ Diagnostic

The main objective of this work is to confirm the affinity and selectivity of 3WW sequences previously engineered and evolved in our Group to bind to HSA and IgG. Therefore, it was (i) expressed GFP in E.coli; (ii) produced an affinity adsorbent A4C7 ([53], for GFP

purification; (iii) Purified GFP and 3GFP fusion proteins; (iv) developed ELISA assays to asses ligands binding to target.

IV.2 - Materials

IV.2.1 - Chemicals

All reagents were used with a high purity and the solvents were pro-analysis.

The reagents for the ligand synthesis, 1-pyrenemethylamine hydrochloride, 95% (A4), Phenylacetic acid (C7), Epichlorohydrin, Isopropyl isocyanide, Sodium thiosulfate ($Na_2S_2O_3$), Sodium periodate ($NaIO_4$), Sodium Chloride (NaCl), ethylene glycol, and the Bicinchoninic Acid (BCA) Kit were acquired from Sigma-Aldrich (Portugal). Methanol (MeOH) and 2-Propanol were obtained from Roth. Dimethylformamide (DMF), Methanol (MeOH), Di- Sodium-hydrogen Phosphate 2-hydrate and Sodium hydroxide (NaOH) were purchased from VWR (Portugal). Ethanol absolute PA, Sodium-di-hydrogen Phosphate 1-hydrate, hydrochloric acid 37% (HCl) were acquired from Panreac (Spain). The glycine used for the elution buffer was acquired from Nzytech.

Human Serum Albumin (HSA), IgG from human serum and Bovine Serum Albumin (BSA) were purchased from Sigma- Aldrich (Portugal). The monoclonal antibody Bevacizumab IgG1 was kindly provided by Prof. Dr. João Gonçalves (Pharmacy Faculty, University of Lisbon, Portugal).

To performed the ELISAS we used Sodium bicarbonate ($NaHCO_3$), Sodium carbonate (Na_2CO_3), ABTS-H_2O_2, Tween 20, Casein from bovine milk acquired from Sigma-Aldrich (Portugal). Soya milk was bought in a supermarket. EZ-Link™ Plus Activated Peroxidase Kit, Thermo Scientific was acquired from VWR (Portugal).

SDS-PAGE was performed using β-mercaptoethanol, glycerol from Aldrich. Tris from Nzytech. 30% acrylamide and bis-acrylamide solution (37.5:1), Sodium dodecyl sulphate (SDS) from Biorad, Ammmonia persulfate (APS), Tetramethylethylenediamine (TEMED), Coomassie Blue R-250 acquired from Roth.

For the bacterial growths, Luria Broth (LB), ampicilin from Nzytech and agar powder from Himedia were used. Protein production was performed using NZY5α competent cells and BL21(DE3) competent cells from Nzytech. The pET 21c expression vector was provided by Dra. Ana Pina (DQ, FCT, UNL). Protein induction was done using Isopropyl β-D-1-thiogalactopyranoside (IPTG) from Nzytech. For DNA purification NZYMiniprep from NZYTech was used. Agarose gels were done using agarose, Tris Base from Nzytech and bromophenol blue

from Roth. Green Safe was used for agarose gel staining and NZYDNA Ladder III was used as molecular weight marker from Nzytech.

IV.2.2 - Chromatographic materials

Agarose SepharoseTM CL-6B was acquired from GE Healthcare; Captiva 96-well filtration block was purchased from Agilent Technologies (USA); Half-area UV-Star® 96-well microplates and 96-well transparent microplates were obtained from Greiner Bio-One (Germany) and Sarstedt (Portugal), respectively. For purification of GFP, WWH4_GFP, WWI8_GFP and WWH18_GFP, was used Tricorn™ empty high performance columns (GE Healthcare, Uppsala, Sweden).

IV.2.3 - Buffers

For the regeneration of the column Regeneration Buffer (0.1M NaOH in 30% isopropanol) was used.The buffer used in the binding and washes for the purifications of WWs and GFP was Phosphate Buffer Saline (PBS) (10mM Sodium phosphate, 150mM NaCl, pH 7.4) in elution step were used 0.1M Glycine-NaOH pH9 or 0.1M Glycine-NaOH pH9 50% ethylene glycol.

To achieve ELISAS it was employed 0.1M carbonate/bicarbonate pH9.6; 10mM Sodium phosphate, 150mM NaCl, pH 7.4, 1% or 5% of soya milk; 10 mM 10mM Sodium phosphate, 150mM NaCl, pH 7.4, 0.05% tween 20, 0.05% (Casein or BSA) for the ELISAS against HSA or IgG, respectively, for the washes and 10 mM 10mM Sodium phosphate, 150mM NaCl, pH 7.4, 0.05% tween 20, 0.5% (Casein or BSA) for the blocking. EZ-Link™ Plus Activated Peroxidase Kit, Thermo Scientific was reconstituted with distilled water, and also 0.1M carbonate/bicarbonate pH9.6 and for quench buffer 3M ethanolamine pH9.

IV.2.4 - Equipment

The synthesis of the synthetic ligands on solid-phase C7A4 was carried out in Incubator ZKA KS4000i (VWR). Temperature control during protein large scale production, microbial pre-inoculum and growth were performed using the Incubator KS4000ic from IKA, Hearaeus multifuge X39 Centrifuge and FrechPress from Thermo Scientific (Portugal). Beackman Couter Optima LE-80K Ultracentrifuge was used for crude extract purification. DNA concentration and purity ratios were calculated using a Nanodrop ND-1000 spectrophotometer Thermo Scientific (Portugal).

The Microplate Reader Tecan Infinite F200 from Tecan was used to perform all the spectrophotometric and spectrofluorometric measurements. Colometric assays were analyzed using a Microplate 96-well Flat bottom Transparent (Starstedt) and in the fluorescence studies, BRAND Plates-Imunograde Tech Scientific were used. Electrophoresis System was acquired from Bio- Rad (Portugal). To obtain the gel image, the GelDoc from Bio- Rad (Portugal) was employed. For purifications AKTA pure (GE Healthcare) was used.

IV.3 - Methods

IV.3.1 - Preparation of ligand C7A4

IV.3.1.1 - Epoxy-activation and functionalization epoxy-activated agarose with aldehyde groups

To synthesize the ligand used in the purification, it was necessary to prepare the agarose so the reaction could occur. In this case the agarose was epoxy-activated and functionalized with aldehyde groups. Procedure similar in Chapter 3 (figure 7). 15g of washed Sepharose$_{TM}$ CL-6B, was epoxyactivated that was ressuspended in 15mL of distilled water and 0.6mL of 10M NaOH after incubated for 30 minutes at 30 º C at 200rpm. After, 1.08mL Epichlorohydrin was added and the mixture was incubated for 3 hours at 36ºC in orbital at 200 rpm. In the end the resin was thoroughly washed and the extension of epoxy-activation was determined through the quantification of hydroxyl groups (OH-). From the epoxy-activation 22 µmol/g of agarose was obtained. For the functionalization with aldehydes, the 15g of epoxy-activated resin was ressuspended in 15mL of 5M of NaOH and incubated overnight at 30°C with orbital shaking at 200 rpm. After, the agarose was washed with distilled water and ressuspended in 15mL of 0.1M of Sodium periodate being incubated at 45°C at 200 rpm for 6 hours. In the end, the functionalized agarose was washed with distilled water.

IV.3.1.2 - Synthesis of the solid phase of Ugi ligand C7A4

In the Ugi reaction the agarose functionalized with aldehyde groups was washed with 20% to 100% methanol (in 20%increments). After that, the amine A4 was added, 5 molar eq. relative to epoxy and incubated for 2h at 60°C with orbital at 200 rpm. After these hours, carboxylic acid compound C7,5 molar eq. relative to epoxy, and Isopropyl isocyanide, 5 molar eq. relative to epoxy was added and the agarose was incubated for 48h at 60°C in orbital at 200 rpm (Figure 26).In the end of the reaction, the resin was washed with: 100% methanol (v/v), 50% methanol (v/v), 50% DMF/distilled water (v/v), distilled water, 0.1M HCl, distilled water, 0.2M NaOH in 50% isopropanol (v/v), 2x distilled water and 20% ethanol (v/v) and stored at 4ºC. In this reaction, the amine, A3, was dissolved in methanol and carboxylic acid, C7, was dissolved in 50% methanol/distilled water (v/v).

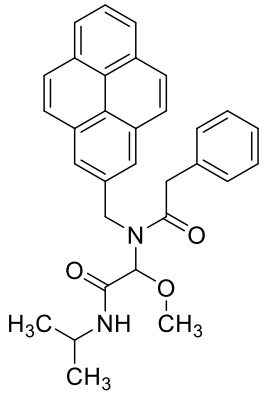


Figure 26 - Structure of affinity ligand C7A4

IV.3.1.3 - Regeneration and equilibration of the ligand

The regeneration (0.1M NaOH in 30% isopropanol and followed by distilled water) of the resin functionalized with the ligand C7A3 was done before the purification of GFP, WWH4_GFP, WWI8_GFP and WWH18_GFP using Tricorn™ high performance columns. After this process, the equilibration was realized with the addition of the 10mM Phosphate Buffer, 150mM NaCl at pH 7.4, the binding buffer.

IV.3.2 - Production of crude extracts containing GFP

IV.3.2.1 - Preparation of Luria Broth (LB) liquid and LB agar medium

The LB medium (25g LB/l distilled water) and LB agar medium (25g LB/l and 15g agar/l) were autoclavated at 120°C for 20min. For LB agar was added ampicilin, the liquid was cooled down at room temperature (RT) and a final concentration of 100µg/ml of ampicilin was added under sterile conditions.

IV.3.2.2 - Transformation of the plasmids pAP006 (GFP) in NZY5α competent cells

In this step, NZY5α competent cells were used to replicate the plasmids. The fragments of DNA was cloned into pET-21c, resulting pAP006 (GFP) provided by Dr. Ana Pina (DQ, FCT, UNL). Then, 50µl of cells and 10µl of plasmid were mixed and incubated in ice for 30min. Afterwards, the solution was subjected to a heat shock during 30s at 42°C, and then incubated

in ice for 2min. After this process, 950µl of LB medium were added to the mixture and it was incubated at 37°C for 1h with constant shaking (210rpm). Afterwards, 100µl of the transformed cells were spread on LB agar ampicilin plates, which were incubated at 37°C overnight. The positive control was performed by adding 1µl of pNZY28 plasmid.

IV.3.2.3 - Isolation and purification of plasmid DNA (pDNA)

For the DNA isolation was used NZYMiniprep kit. On the previous day, the pre-inoculum was performed by using test tubes containing 6ml of LB medium, 100mg/ml of ampicilin and one colony of the corresponding freshly transformed plate (obtained in IV.3.2.2), which was incubated overnight at 37°C at 210rpm. The resulting cultures were centrifuged for 2min at 11000×*g*, and the supernatant was removed.

The cell pellet was ressuspended in 500µl of buffer A1/RNAse by vortexing. 500µl of buffer A2 was added, the solution was mixed and it was incubated at RT for 4min. In the end, 600µl of buffer A3 was added and gently mixed, the solution was centrifuged for 10min at 11000×*g* at RT. The supernatant was loaded into a NZYTech spin column placed inside a 2ml collecting tube. It was centrifuged 2min at 11000×*g* and the flow-through was discarded. The spin column was washed by adding 500µl of buffer AY, centrifuged 2min at 11000×*g*, added 600µl of buffer A4 and centrifuged again. The flow-through was discarded, and the column was centrifuged again for 3min at 11000×rpm. For the pDNA elution, the column was placed inside a new collecting tube and 30µl of deionized warm water was added to the column and incubated at 65°C for 1min. The column was centrifuged for 2min at 11000×*g,* the obtained flow-through corresponded to the first elution of pure pDNA. A second elution with 50µl of deionized warm water was performed in the same conditions .In the end of the isolation and purification the plasmid concentration was determined.

IV.3.2.4 - Evaluation of pDNA through agarose gel electrophoresis

For this step, 0.8% agarose gel was prepared by dissolving the agarose in 100ml of TAE pH8.5 (40mM Tris-base, 20mM glacial acetic acid, 1mM EDTA) and the solution was poured into the casting frame with the comb inserted. The agarose gel was polymerized for 1h and transferred to the running module. The tank was filled with TAE and the samples were applied in each well.

The running conditions were set as 100V for 1h. Afterwards the gel was immersed in the staining solution (100ml TAE, 10µl Green Safe) for 30min and photographed. The solutions for

loading were prepared as followed: 2µl of sample or 5µl of the molecular weight marker and 5µl of loading buffer (65% sucrose, 10mM Tris-HCl, 10mM EDTA, 0.3% bromophenol blue).

IV.3.2.5 - Transformation of BL21(DE3) competent cells

For the BL21(DE3) transformation competent cells were used for protein expression of T7 RNA polymerase based systems, in this case the protocol was the same used in transformation of the plasmids pAP006 (GFP) in NZY5α competent cells.

IV.3.2.6 - Protein expression in BL21(DE3) competent cells

Pre-inoculum was performed, in the previous day, as described IV.3.2.2 with the resulting transformant colonies from IV.3.2.5. After 7h of incubation at 37°C (210rpm), 1ml of the culture was used to inoculate 50ml of LB medium containing 0.1µg/ml ampicillin. The culture was incubated overnight at 37°C (210rpm).

The next day, 10ml of the resulting culture was used to inoculate 1L of LB medium containing 0.1µg/ml ampicillin, and it was incubated at 37°C in orbital shaker. The culture growth was monetarized by optical density at 600nm. Once the cultures reached OD_{600nm} 0.6-0.8 the protein expression was induced by adding 1ml of IPTG (1M). The protein expression was monetarized by GFP fluorescence, OD_{600nm} and SDS-PAGE analysis.

IV.3.2.7 - Cellular fractionation

After 24h of protein expression, the cell culture was centrifuged (20min, 18000×*g* at 4°C). The ressuspended cells in 10mM Sodium phosphate, 150mM NaCl, pH 7.4 were subjected to three freeze/thaw cycles. After, the cell lysis was performed by a mechanical process (French Press), with a maximum of 20000psi of pressure applied. Afterwards, DNAseI was added, the sample was incubated for 30min in ice and centrifuged (15min, 18000×*g* at 4°C). The resulting pellet was ressuspended in 15ml of PBS and stored at -20°C. The supernatant was further ultracentrifuged at 180000×*g* for 1h30 at 4°C. The protein expression was monitorized by GFP fluorescence and SDS-PAGE analysis.

IV.3.3 - Protein quantification methods

IV.3.3.1 - Sample preparation for SDS-PAGE analysis

To evaluate the amount of protein expression, sample volumes were normalized. The normalization was performed according to the ratio $1.2/OD_{600nm}$ for each sample. The corresponding volumes were centrifuged 5min at 6800×*g* and the supernatant was discarded. All samples were ressuspended in 20µl of sample buffer and boiled for 2min. 15µl of each sample was used to apply in the 12.5% acrylamide/bisacrylamide gel.

IV.3.3.2 - SDS-PAGE preparation

In this work, 12.5% acrylamine/bisacrylamine SDS-PAGE gels, described in III.3.3.1 (Chapter 3), were used..

IV.3.3.3 - Coomassie Blue staining

After the running, the SDS-PAGE gel was employed into the staining solution (1g Coomassie Blue R-250, 15 mL glacial acetic acid, 90 mL methanol and 95 mL distilled water) for 30 min with agitation. Then, the gel was placed in a destaining solution (75 mL glacial acetic acid, 450 mL methanol and 475 mL distilled water) overnight.

IV.3.3.4 - Purification of GFP, WWH4_GFP, WWI8_GFP and WWH18_GFP

Ligand C7A4 was packed in Tricorn™ high performance columns (3g). The Binding Buffer was Phosphate Buffer Saline (PBS) (10mM Sodium phosphate, 150mM NaCl, pH7.4) and the Elution Buffer was 0.1M Glycine-NaOH pH9 or 0.1M Glycine-NaOH pH9 50% ethylene glycol using AKTA pure. The samples were collected in a 96-well microplate.

IV.3.3.5 - Quantification of GFP and total protein

The quantification of GFP, WWH4_GFP, WWI8_GFP and WWH18_GFP by pipetting 200µL of each samples in a 96-well microplate and measuring the fluorescence intensity (λexcitation=485nm and λemission=535nm). A calibration curve was obtained using a pure GFP solution (10-6-10-1mg/ml). PBS was used as blank. The total protein was quantified through the colorimetric BCA assay described in Chapter 3. 25µl of each samples were added to each well in a transparent 96-well microplate, followed by the addition of 200µl of prepared BCA reagent. The microplates were incubated at 37°C during 30min and the absorbance at 560nm was measured. A calibration curve was obtained using a solution of BSA (0-1mg/ml).

IV.3.4 - Binding Studies

IV.3.4.1 - ELISA assay between WWH18_GFP and HSA

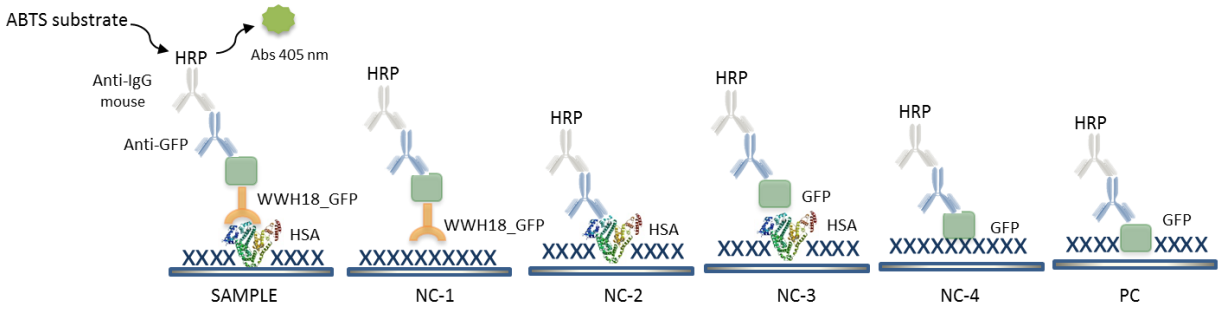


Figure 27 - Binding between WWH18 or WWH4 using 1ug/well of each protein. As ELISA controls were used: NC-1-without target only blocking solution and WWs was added; Controls- GFP (1ug/well) without WWs was used as negative control. (i) NC-3 (HSA) was immobilized in the well; (ii) PC (no HSA), well without target, the GFP solution was immobilized in the well and the well was blocked; (iii) NC-4 (no HSA), equal to NC-3. In the end, NC-2 (HSA), the target was immobilized In the well, however no GFP protein was added, the well was blocked and the detection proceed with the primary antibody (Anti-GFP) and the secondary (Anti-IgG mouse).

ELISA tests were performed in triplicate, and included four negative controls and one positive control. The first negative control (NC-1) well without a target where it was added the WWH18_GFP, after which the wells were blocked; the second negative (NC-2) was the GFP immobilized (1µg/well) without the WWH18_GFP; the third negative (NC-3) HSA (1µg/well) immobilized with GFP protein (1µg/well) instead of WWH18_GFP; the third negative (NC-4) was verified after the blocking was added GFP (1µg/well), this negative is not the target (HSA)

The binding wells were incubated overnight at 4°C with the target HSA (1µg/well), the solution was prepared in coating buffer (0.1M carbonate/bicarbonate pH 9.6) (100µL). After this time, the plate was wash (PBS pH 7.4 1x), the blocking solution was added (PBS 1 % Soya Milk), which was incubated for 1h at room temperature. The wells were washed with (PBS pH 7.4 1x), and the WWH18_GFP ((1µg/well) was added, which was incubated for 2h at room temperature. After this time the wells were washed using PBS 0.05% Tween 20 (5x) and 1 time with PBS pH7.4. Then, it 200µL solution of the anti-GFP antibody dilution 1:200 in PBS 1% Soya Milk was added, and incubated 1h at room temperature. The next step was washing the wells to remove unbound antibody, with 5 times of PBS. After the second antibody was added, conjugate anti-mouse IgG-HRP diluted 1:1000 in PBS 1% Soya Milk and incubated 1h at room temperature. In the end, the detection occurred using 200µL of a substrate solution ABTS-H_2O_2 that was incubated in the dark for 60 minutes at 37°C, and for analyses, the Abs405nm was read.

After this process was optimized the conditions of the ELISA and the time that each step occurs.

IV.3.4.2 - ELISA assay between WWH4_GFP and HSA

The procedure for ELISA assay between WWH4_GFP and HSA was similar to procedure described in IV 3,4,1, with the optimized conditions.

The binding wells were incubated overnight at 4°C with the target HSA (1µg/well), the solution was prepared in coating buffer (0.1M carbonate/bicarbonate pH 9.6) (100µL). Then, the plate was washed (10mM PBS, 0.05%Tween 20, 0.05% casein pH7.4; 3x), a blocking solution was added (10mM PBS, 0.05%Tween 20, 0.5% casein pH7.4), which was incubated for 2h at room temperature. The wells were washed with (10mM PBS, 0.05%Tween 20, 0.05% casein pH7.4; 3x), and the WWH18_GFP/WWH4_GFP (1µg/well) added, which was incubated for 2h at room temperature. Afterwards, the wells were washed using 10mM PBS, 0.05%Tween 20, 0.05% casein pH7.4 (5×) and 1 time with PBS pH7.4. Then, a 100µL solution of the anti-GFP antibody dilution 1:200 in 10mM PBS, 0.05%Tween 20, 0.05% casein pH7.4 was added, and incubated 1h at room temperature. The next step was wash the wells to remove unbound antibody, with 5 times of PBS pH7.4. After the second antibody was added (100µL), conjugate anti-mouse IgG-HRP diluted 1:1000 in 10mM PBS, 0.05%Tween 20, 0.05% casein pH7.4 and incubated 1h at room temperature. In the end, the detection occurred using 100µL of a substrate solution ABTS-H_2O_2 that was incubated in the dark for 60 minutes at 37°C, and for analyses, the Abs_{405nm} was read.

IV.3.4.4 - ELISA assay between WWI8_GFP and IgG

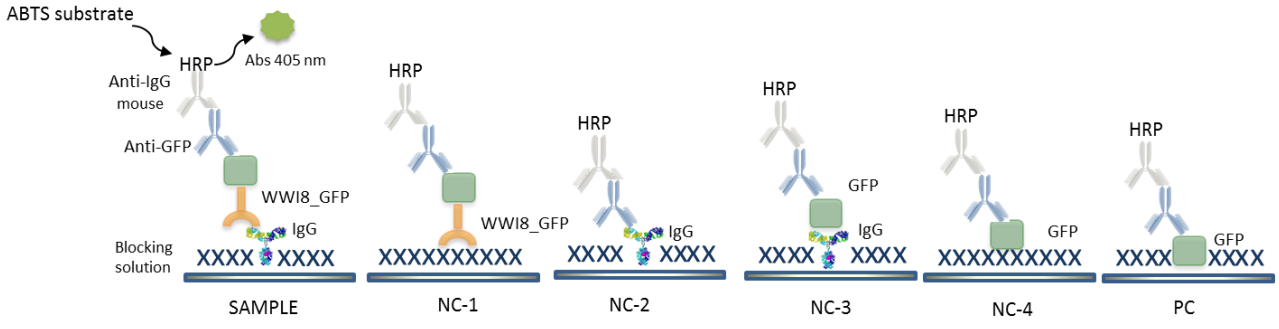


Figure 28 - Binding between WWI8 using 1ug/well of each protein. ELISA controls were used: NC-1- without target only blocking solution and WWs was added; Controls- GFP (1ug/well) without WW was used as negative control. (i) NC 3 (IgG) was immobilized in the well; (ii) PC (no IgG), well without target, the GFP solution was immobilized in the well and the well was blocked; (iii) NC-4(no IgG), equal to NC-3. In the end, NC-2 (IgG), the target was immobilized In the well, however no GFP protein was added, the well was blocked and the detection proceed with the primary antibody (Anti-GFP) and the secondary (Anti-IgG mouse).

For this study, different approaches were used, the casein was substituted by BSA and the polyclonal antibody IgG was substituted by monoclonal antibody Bevacizumab IgG1 that was kindly provided by Prof. Dr. João Gonçalves (Pharmacy Faculty, University of Lisbon, Portugal).

IV.3.4.5 - Conjugation of human IgG and HSA with horseradish-peroxidase (HRP)

EZ-Link™ Plus Activated Peroxidase Kit (1mg) was reconstituted in 100µl of distilled water and chilled on ice. 0.5 mg of proteins (HSA and IgG) was reconstituted in 50µl of conjugation buffer (0.1M carbonate/bicarbonate) and added to the enzyme solution (divided in two 50µl for each protein). The mixture was incubated overnight at 0-4°C. After this time, 30µl of quenching buffer (3M ethanolamine pH9) was added, and the solution was incubated for 2h at room temperature. In the end, 350µl of distilled water was added and the solution dialyzed against PBS buffer (10mM Sodium phosphate, 150mM NaCl pH7.4).

IV.3.4.6 - Direct ELISA assay

The plate was coated with 100 µL of WWs (WWH18_GFP, WWH4_GFP and WWI8_GFP), (1µg/mL) in coating buffer (0.1M carbonate/bicarbonate pH9.6), overnight at 0-4°C. Then, it was washed 3 times with 10mM Sodium phosphate, with 10mM Sodium phosphate, 150mM NaCl, 0.05%casein, 0.05%Tween 20 pH7.4 (300µL) and was blocked with 10mM Sodium phosphate, 150mM NaCl, 0.5%casein, 0.05%Tween 20 pH7.4 by adding 200µL/well and incubating for 2h at room temperature. In the end, the plate was extensively washed with 10mM Sodium phosphate, 150mM NaCl, 0.05%casein, 0.05%Tween 20 pH7.4 (5× 300µL each) and PBS pH7.4 (1× 300µL each). Afterwards, 100µL/well HSA-HRP and IgG-HRP dilute 1:200 were added dilute 1:200 to the respective coating protein. The plate was washed in 10mM Sodium phosphate, 150mM NaCl (3× 300µL each). The detection occurred using 100µL of a substrate solution ABTS-H_2O_2 that was incubated in the dark for 60 minutes at 37°C, and for analyses, the Abs_{405nm} was read. This test was performed in triplicate with two negative controls and one with sample: (i) the samples where the WWs were coated (1µg/mL) in coating buffer (0.1M carbonate/bicarbonate pH9.6), were blocked with 10mM Sodium phosphate, 150mM NaCl, 0.5%casein, 0.05%Tween 20 pH7.4 and after the respective WWs were exposed to the IgG-HRP or HSA-HRP as target. (ii) In the negative control, the plate was only blocked and was added the target, (iii) the other negative control was coated with GFP, blocked and after was added the target.

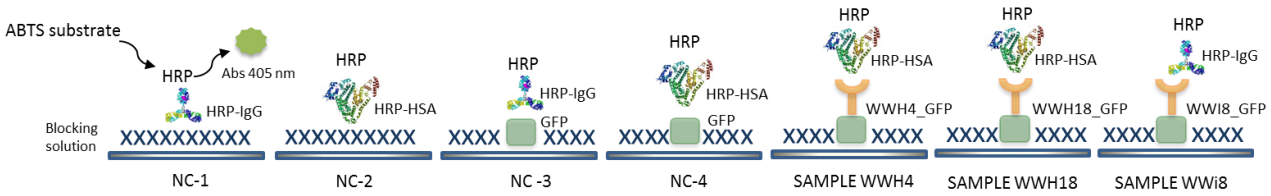


Figure 29 - Direct assay. ELISA controls were used: NC-1-without target only blocking solution and HSA or IgG-HRP (NC-2) was added; Controls- GFP (1ug/well) without WW was used as negative control. (i) NC 3 GFP was immobilized in the well and was added HSA-HRP or IgG-HRP (NC-4); Samples were incubated overnight with WWs (WWH4, WWH18, WWI8) and after blocking were added HSA-HRP or IgG-HRP.

IV.4 - Results and discussion

This Chapter was a continuation of a previous work realized in the Biomolecular Engineering Lab by Ana Margarida Dias (PhD). The first part of the work was to develop two affinity protein libraries, based on ribosome display. From the screening against HSA and IgG with these libraries resulted three protein sequence, two specificity for HSA and one specify for IgG.

To execute the test that confirm the specificity of the proteins selected it was necessary to produce and purify the proteins used on the Enzyme-Linked Immunosorbent Assay (ELISA).

IV.4.1 - Fragment DNA design and DNA purification

The plasmid pAP006, plasmid that express GFP, was provided by Dra. Ana Pina (DQ, FCT, UNL. This plasmid was amplified in NZY5α competent cells. Afterwards, the plasmid DNA (pDNA) was isolated using the isolation NZYMiniprep kit and the final purity and concentration was determined by spectroscopic analysis (Nanodrop) (Table 15).

Table 15 - pDNA concentration and purity of the plasmids used for the large scale production of GFP

Plasmid (protein encoded)	Elution	DNA concentration (ng/µl)	Purity	
			A_{260}/A_{280}	A_{260}/A_{230}
pAP006 (GFP)	1st	64	1.97	2.76
	2nd	11.9	1.7	26.63

The isolation of the plasmid resulted in the successful recovery of pDNA with high concentration and purity for the first elution step. The DNA and RNA absorb at 260nm and proteins at 280nm. As described in technical support bulletin of NanoDrop, a ratio of 1.8 is generally accepted as “pure” DNA. The resulting pDNA of the first elution was used to transform BL21(DE3) cells to obtain final crude extracts containing the protein of interest.

IV.4.2 - Large scale production of GFP

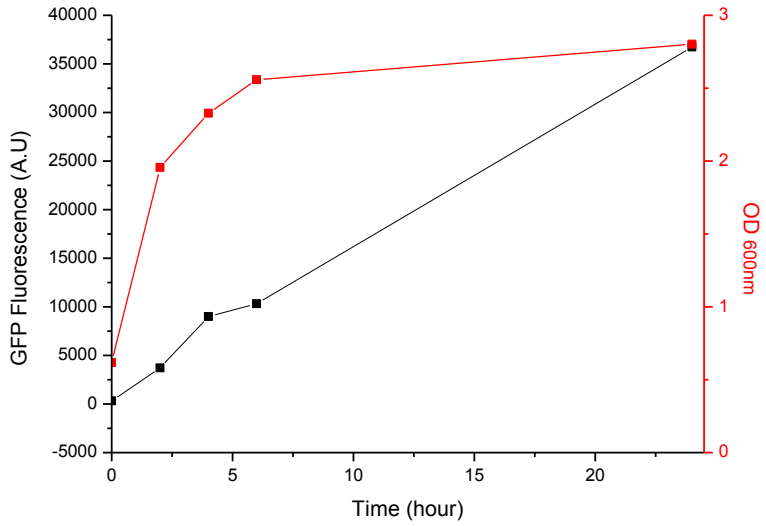


Figure 30 - GFP fluorescence (black) and optical density measurements (red) after the induction (t=0) during the large scale (1L) expression of GFP

Previous studies showed the optimal conditions for the large scale production of GFP in BL21(DE3) cells , and so, the same conditions were followed. Protein production was monitored by fluorescence intensity and OD_{600nm} measurements (Figure 3), and there is a correlation between the two measurements with the increasing induction time. The amount of protein produced by the cells increases with time after induction.

Afterwards, the cells were harvested and lysed to obtain the final crude extract used in the purification of the protein of interest.

IV.4.3 - Purification of the crude extract to obtain GFP, WWH4_GFP, WWI8_GFP and WWH18_GFP

For purification of GFP, WWH4_GFP, WWI8_GFP and WWH18_GFP,(domains with GFP tag to facilitate in purification with affinity ligand C7A4 [53]) was used Tricorn™ high

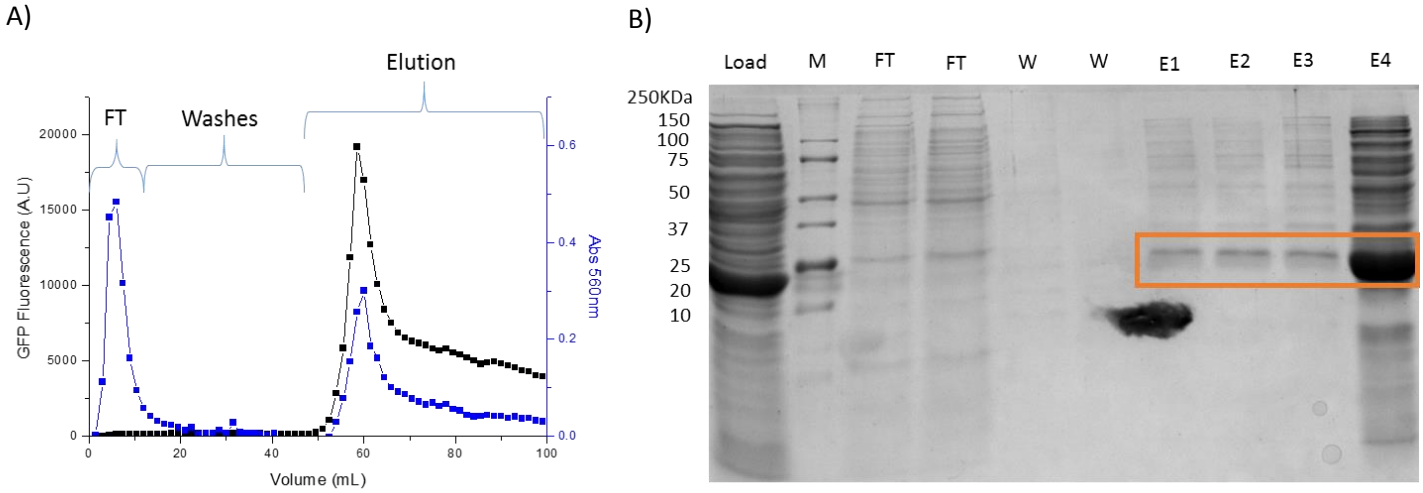


Figure 31 - (A) Chromatogram with fluorescence of GFP and BCA (560nm) of washes and elution; (B) SDS-PAGE gel obtained for GFP purification with C7A4 affinity ligand. The molecular marker, loading, pure HSA, flow-through, washes and elution fractions correspond to M, L,HSA, FT, W and E, respectively.

performance columns with 3g of C7A4 in the AKTA pure. Ligand design for purification of the GFP [53]. The flow-through and washes were realized with 10mM Sodium phosphate, 150mM NaCl pH7.4 and the elution was done with 0.1M Glycine-NaOH 50%ethylene glycol pH9

This is confirmed by the SDS-PAGE analysis, as shown in Figure 4. GFP has a molecular weight of approximately 29kDa [64].

In this chapter, it was also needed to purify the WW prototypes, each with a different estimated molecular weight KDa, and with diferent sequence of amino acids in region of loop, these results were previously obtained by Ana Margarida Dias, PhD, the data is summarize in Table3.

Table 16 - Comparison between the fusions WW with GFP, selected by ribosome display. In green are the amino acids that take part in loops and that were randomized. In bold are the amino acids that are in limits of the loop.

WW prototype	Estimated MW (KDa)	Amino acid sequence	Size	Target
H4	35.6	SMGLPPGWDEYK**T**RLWYW**G**KTYYYN**H**WT DN**K**TSTWTDPRMSS	42	HSA

H18	38.3	SMGLPPGWDEYK**T**FWCLS**G**KTYYYN**H**WW WS**K**TSTWTDPRMSS	42	HSA
I8	33.1	SMGLPPGWDEYK**T**WLWAL**G**KTYYYN**H**GCR R**K**TSTWTDPRMSS	42	IgG

In case of WWH4_GFP the flow-through and washes were performed with 10mM Sodium phosphate, 150mM NaCl pH7.4, whereas the elution was done with 0.1M Glycine-NaOH pH9 (elution E1-E3). However, it is possible to see in Figure 32B from E1 to E3 the percentage of protein eluted was minimum comparatively with the loading, indicating that elution did not work. In this case, a new buffer was added to the column 0.1M Glycine-NaOH 50%ethylene glycol pH9; before the quantification was done, it was needed to change the buffer to 10mM Sodium phosphate, 150mM NaCl pH7.4.

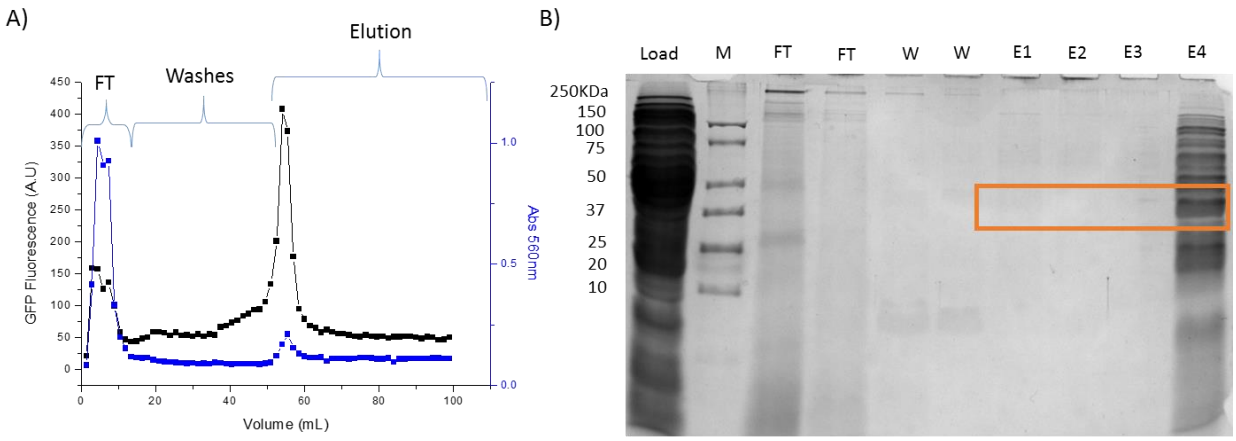


Figure 32 - A) Chromatogram with fluorescence of GFP and BCA (560nm) of washes and elution; (B) SDS-PAGE gel obtained for WWH4_GFP purification with C7A4 affinity ligand. The molecular marker, loading, pure HSA, flow-through, washes and elution fractions correspond to M, L,HSA, FT, W and E, respectively.

On the contrary, the WWH18_GFP with HSA as target, was eluted with the same conditions, not being necessary to change the elution buffer for ethylene glycol (Figure 33).

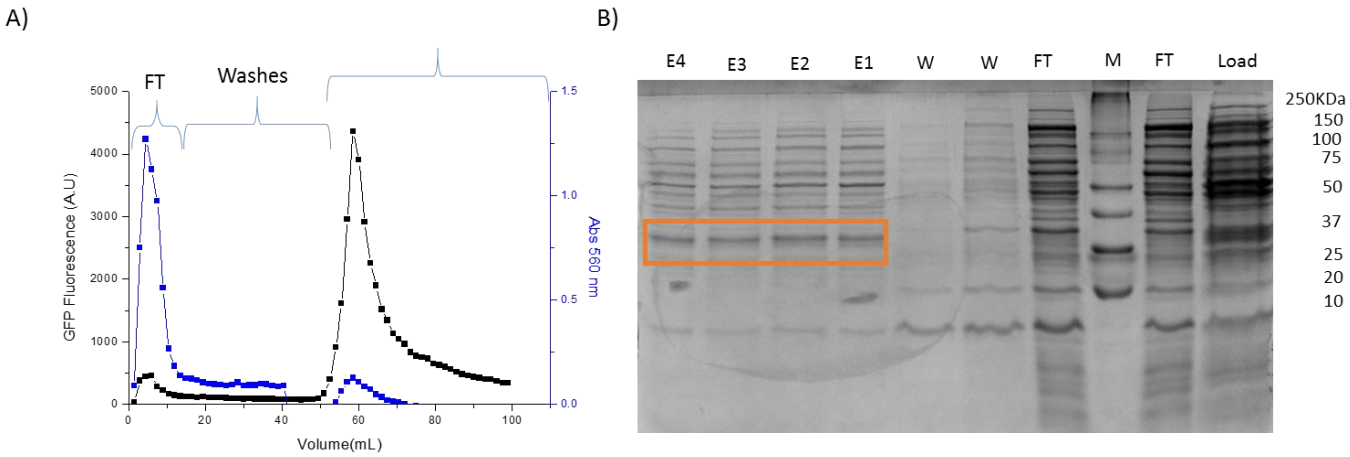


Figure 33 - (A) Chromatogram with fluorescence of GFP and BCA (560nm) of washes and elution; (B) SDS-PAGE gel obtained for WWH18_GFP purification with C7A4 affinity ligand. The molecular marker, loading, pure HSA, flow-through, washes and elution fractions correspond to M, L,HSA, FT, W and E, respectively.

In the end the WW prototype whose target is IgG (WWI8_GFP) (Figure 34) was purified For flow-through and washes a 10mM Sodium phosphate, 150mM NaCl pH7.4 was used and the elution was done with 0.1M Glycine-NaOH pH9.

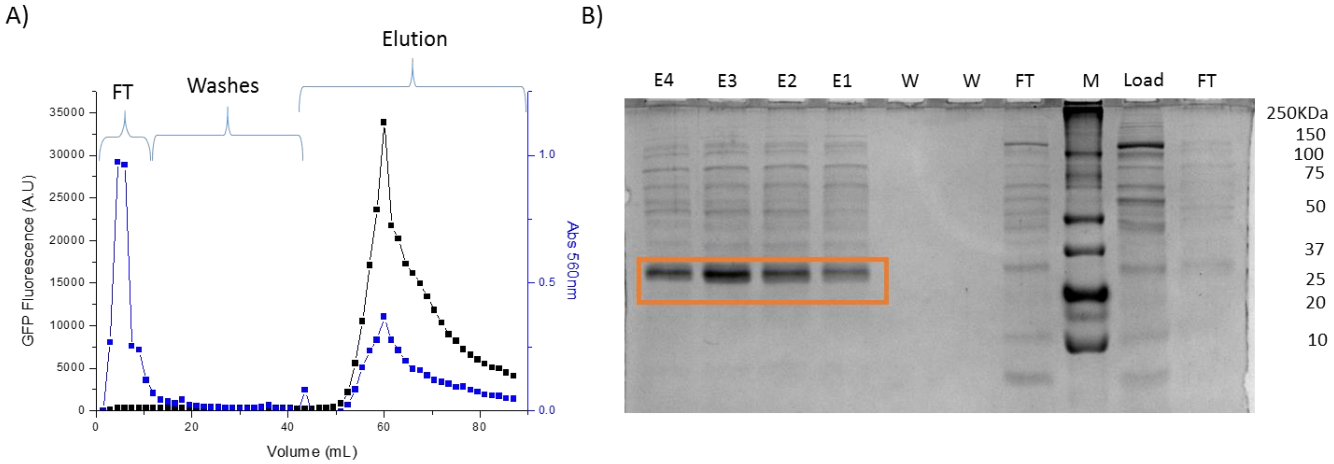


Figure 34 - A) Chromatogram with fluorescence of GFP and BCA (560nm) of washes and elution; (B) SDS-PAGE gel obtained for WWHI8_GFP purification with C7A4 affinity ligand. The molecular marker, loading, pure HSA, flow-through, washes and elution fractions correspond to M, L,HSA, FT, W and E, respectively.

Despite having some protein percentage in flow-through and washes, for all purified proteins, the most of it leaves during the elution step.

Afterwards, with GFP, WWH4_GFP, WWI8_GFP and WWH18_GFP purified, was proceeded the realization of ELISAS for tested the specificity of proteins to the target that was designed.

IV.4.4 - Results from ELISAs and optimization of process

The Enzyme-Linked Immunosorbent Assay was prepared in order to get the whole picture of interaction possibilities. The main goal was to observe the possible interactions between proteins and their selected targets..

The first ELISA used the protein WWH18_GFP whose target was HSA. In this ELISA four negative controls were performed, one positive and one with the sample. This controls helps to monitor the process so that the optimization could be executed..

In this test, PBS was used with 1% Soya Milk for blocking and diluting the antibody's and 0.1M carbonate/bicarbonate pH9.6 was used for the coating of the proteins. In the end, the reaction was followed by reading at 405 nm by the transformation of ABTS substrate from HRP. The results are summarized in the following Figure.

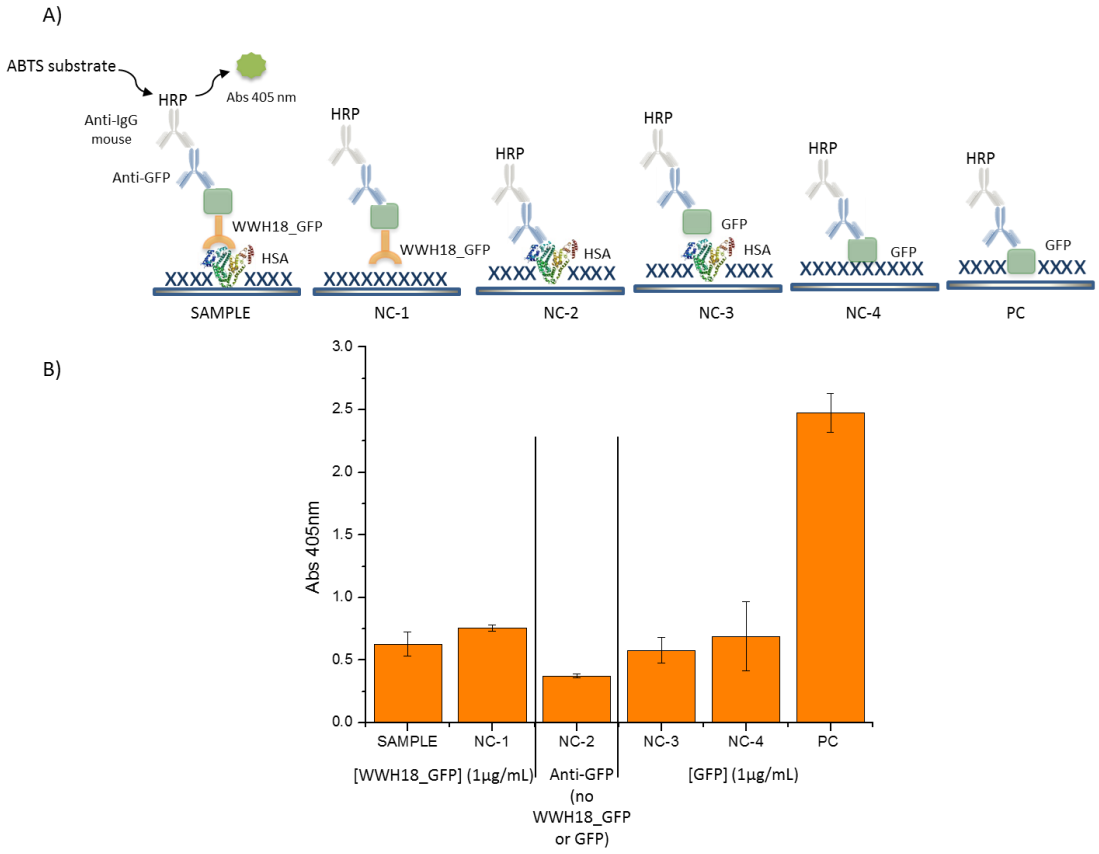


Figure 35 - (A) Binding between WWH18 using 1ug/well of each protein. As ELISA controls were used: NC-1-without target only blocking solution and WWs was added; Controls- GFP (1ug/well) without WWs was used as negative control. (i) NC-3 (HSA) was immobilized in the well; (ii) PC (no HSA), well without target, the GFP solution was immobilized in the well and the well was blocked; (iii) NC-4 (no HSA), equal to NC-3. In the end, NC-2 (HSA), the target was immobilized In the well, however no GFP protein was added, the well was blocked and the detection proceed with the primary antibody (Anti-GFP) and the secondary (Anti-IgG mouse).(B) Results from the test (n=3).

The proteins GFP, HSA and WWH18_GFP were placed with 1µg/mL in each well and the test was made in triplicate. The negative controls were realized to nullify all possibilities of false signals, in two controls was blocked with PBS 1% Soya Milk and then placed the WWH18_GFP or GFP. In the other two negative controls the plate was coating overnight with HSA, then was added GFP to one and the other does not put anything calling itself anti-GFP well. The positive controls was used for see if systems work.

With this results was possible observe that the negative have the same Abs 405 nm that the samples and concludes that possible the solution of blocking was not enough to block the adsorption the other proteins to the plate. In this case was optimized the solutions of blocking, washes. In the next table are summarize the optimization and the times that each phase occurs.

Table 17 - Protocol used for the optimization of ELISA

H18	Sample	NC (no HSA)	NC (HSA)	PC (no HSA)	NC (no HSA)	NC anti-GFP
Coating (Overnight)	HSA (1ug/well) 100 uL *	0.1M carbonate/bicarbonate pH 9.6 100 uL	HSA (1ug/well) 100 uL *	GFP (1ug/well) 100 uL *	0.1M carbonate/bicarbonate pH 9.6 100 uL	HSA (1ug/well) 100 uL*
Wash	PBS 0.05% tween 20 + 0.05% casein					
Blocking (2h@25ºC)	PBS 0.05% tween 20 + 0.5% casein					
Wash	PBS 0.05% tween 20 + 0.05% casein					
Protein (2@25ºC)	WWH18-GFP (1ug/well) 100 uL **	WWH18-GFP (1ug/well) 100 uL **	GFP (1ug/well) 100 uL **	PBS 100 uL	GFP (1ug/well) 100 uL **	PBS 100 uL
Wash	4 x PBS 0.05% tween 20 + 0.05% casein 1 x PBS					
1º antibody (1h@25ºC)	Anti-GFP antibody (1:200) PBS 0.05% tween 20 + 0.05% casein (100 uL)					
Wash	5 x PBS 0.05% tween 20 + 0.05% casein					
2º antibody (1h@25ºC)	Anti-IgG mouse antibody (1:1000) PBS 0.05% tween 20 + 0.05% casein (100 uL)					
Wash	5 x PBS 0.05% tween 20 + 0.05% casein 1 x PBS					
Substrate (1h@37ºC)	ABTS-H2O2 (100 uL)					

- 0.1 M carbonate/bicarbonate pH 9.6

** PBS

This optimization was realized for the WW prototype, WWH18_GFP as WWH4_GFP, and the results are in Figure 36.

In this case was possible see that the ELISA was successful, the negative controls are with low absorbance and the positive are the maximum, in other words the system work, however both of WW not bind to HSA.

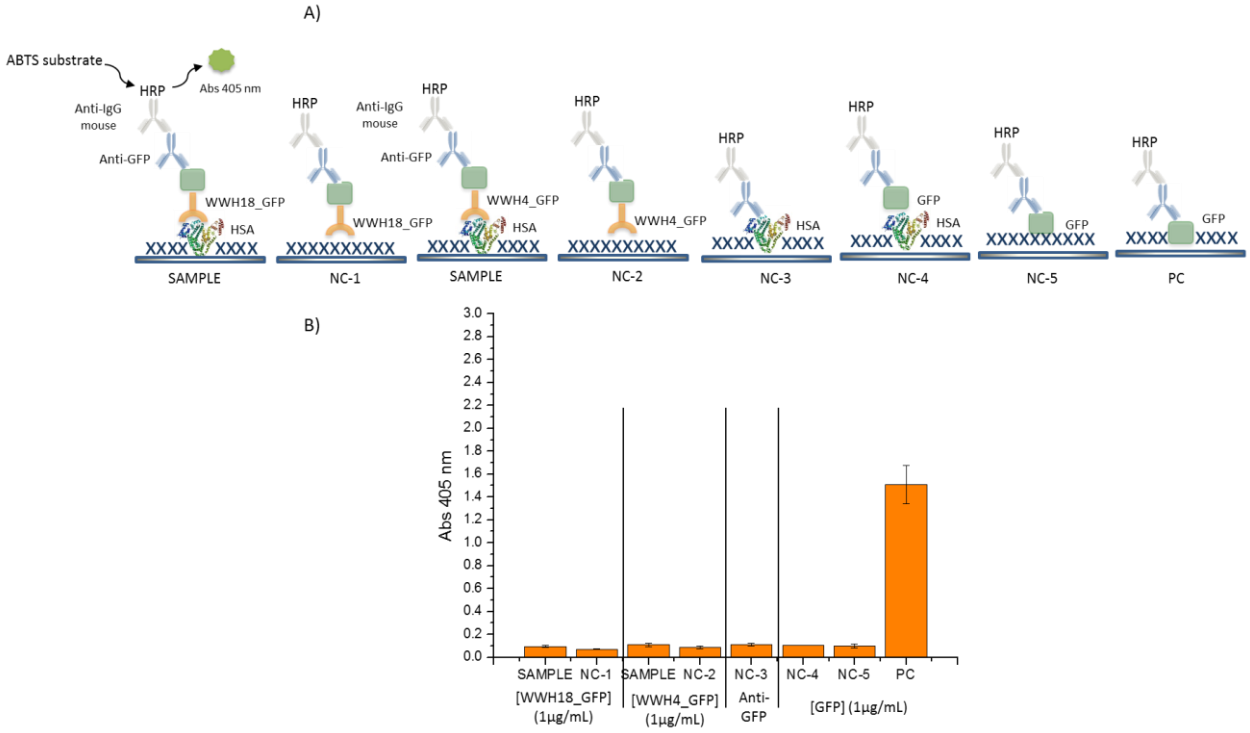


Figure 36 - (A) Binding between WWH18 or WWH4 using 1ug/well of each protein. As ELISA controls were used: NC-1/NC-2-without target only blocking solution and WWs was added;; Controls- GFP (1ug/well) without WWs was used as negative control;(i) PC (no HSA), well without target, the GFP solution was immobilized in the well and the well was blocked;(ii) NC-4 (HSA) was immobilized in the well (iii) NC-5 (no HSA), equal to NC-4. In the end, NC-3 (HSA), the target was immobilized In the well, however no GFP protein was added, the well was blocked and the detection proceed with the primary antibody (Anti-GFP) and the secondary (Anti-IgG mouse).(B) Results from the test (n=3).

Afterwards, was testes the same system for WWI8_GFP against IgG. To avoid cross reactivity was used BSA instead of casein, this was derivate from milk from bovine and could possess immunoglobulins (Figure 37).

In situation of this ELISA the negative are to sign, in this case the negative control Anti-GFP gives signal which may indicate that possible exist cross reactivity between the Anti-GFP antibody or Anti-IgG antibody from mouse with the IgG adsorbed in plate. To address this problem was used the monoclonal antibody Bevacizumab IgG1 kindly provided by Prof. Dr. João Gonçalves (Pharmacy Faculty, University of Lisbon, Portugal). In other hand, with the other negative controls was possible conclude that possible the step of blocking was not succeeded so was used casein instead of BSA.

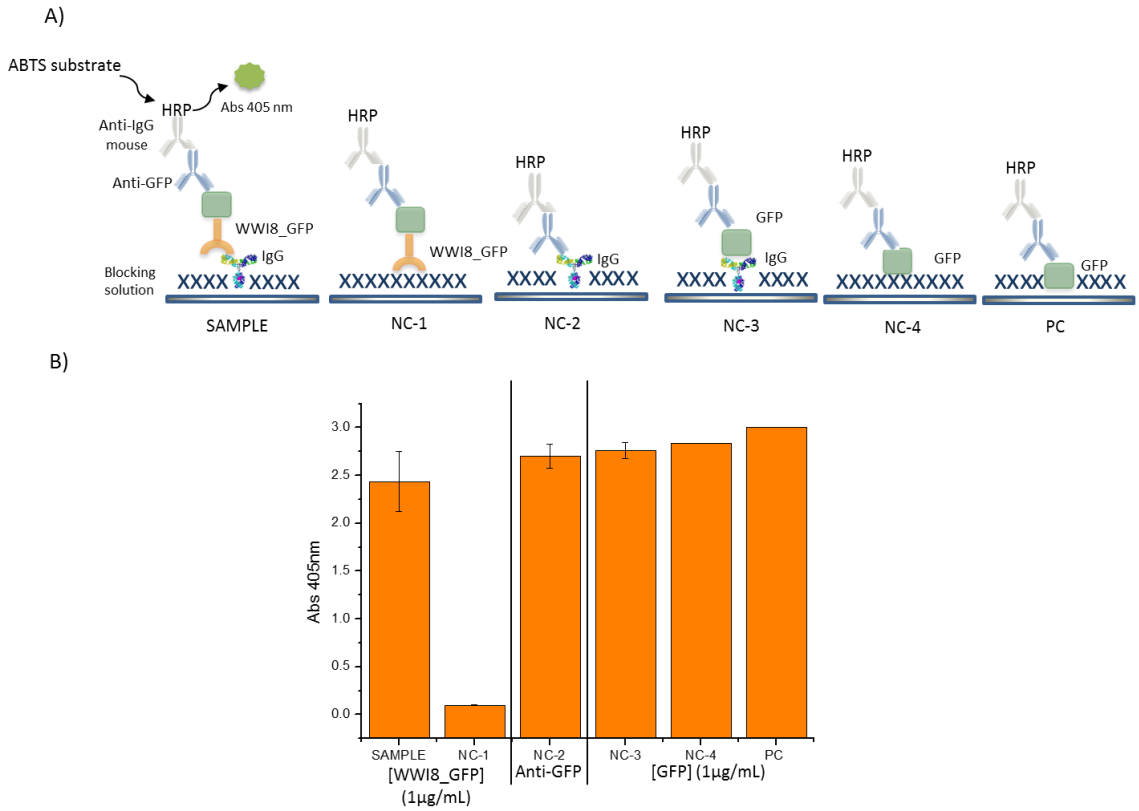


Figure 37 - (A) Binding between WWI8 using 1ug/well of each protein. ELISA controls were used: NC-1- without target only blocking solution and WWs was added; Controls- GFP (1ug/well) without WW was used as negative control. (i) NC 3 (IgG) was immobilized in the well; (ii) PC (no IgG), well without target, the GFP solution was immobilized in the well and the well was blocked; (iii) NC-4(no IgG), equal to NC-3. In the end, NC-2 (IgG), the target was immobilized In the well, however no GFP protein was added, the well was blocked and the detection proceed with the primary antibody (Anti-GFP) and the secondary (Anti-IgG mouse). (B) Results from the test (n=3).

The results from the new ELISA with the new alternatives are represent in Figure 38. With this test it was possible to observe that the positive control does not have signal, and the well correspondent to the Anti-GFP have absorbance at 450nm concludes that there is possibly cross reactivity between the antibodies. Nevertheless, the sample have signal, on the other hand the negative control does not have absorbance, negative with the protein of interest WWI8_GFP. Therefore, it is interesting to proceed with this WW prototype for another test, the direct ELISA assay. In this case was necessary to conjugate the HSA and IgG with horseradish-peroxidase (HRP). The test was also realized with the other WW prototypes.

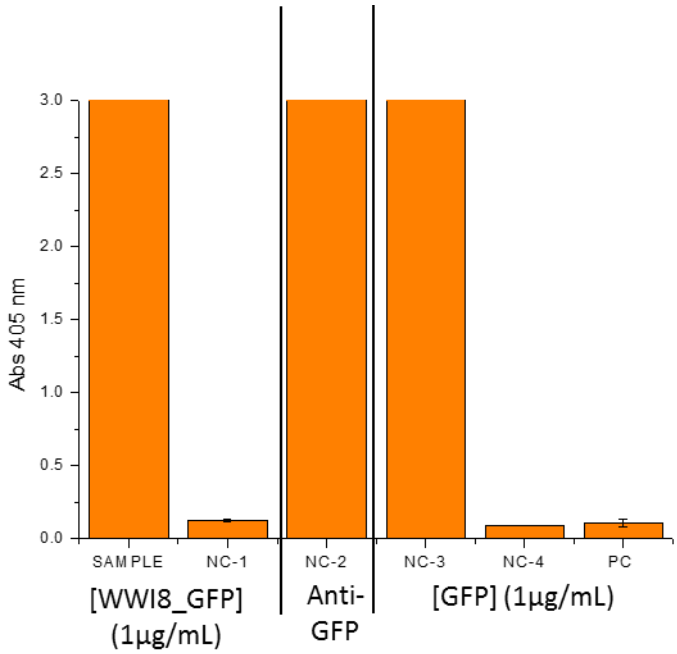


Figure 38 - Results from ELISA of WWI8_GFP agaist IgG, using Bevacizumab IgG1 and casein for blocking.

In this test, three approaches were considered: (i) the samples where the WWs were coated (1µg/mL) in coating buffer (0.1M carbonate/bicarbonate pH9.6), were blocked with 10mM Sodium phosphate, 150mM NaCl, 0.5%casein, 0.05%Tween 20 pH7.4 and after were exposed to the IgG-HRP or HSA-HRP as the target for which were selected.(ii) in this approach the plate was only blocked and was added the target.(iii) the other was coated GFP, blocked and after was added the target.

From the results it was possible to conclude the non existence selectivity of WWH4, WWH18, WWI8 for HSA or IgG. In this test all the approaches are negative (no transformation of ABTS substrate from HRP). Since the positive control was not done, it is not possible to understand if the problem is in formation of HRP-IgG or HRP-HSA as this undergoes dialysis. The next step would be the realization of the positive control to examine whether the system works.

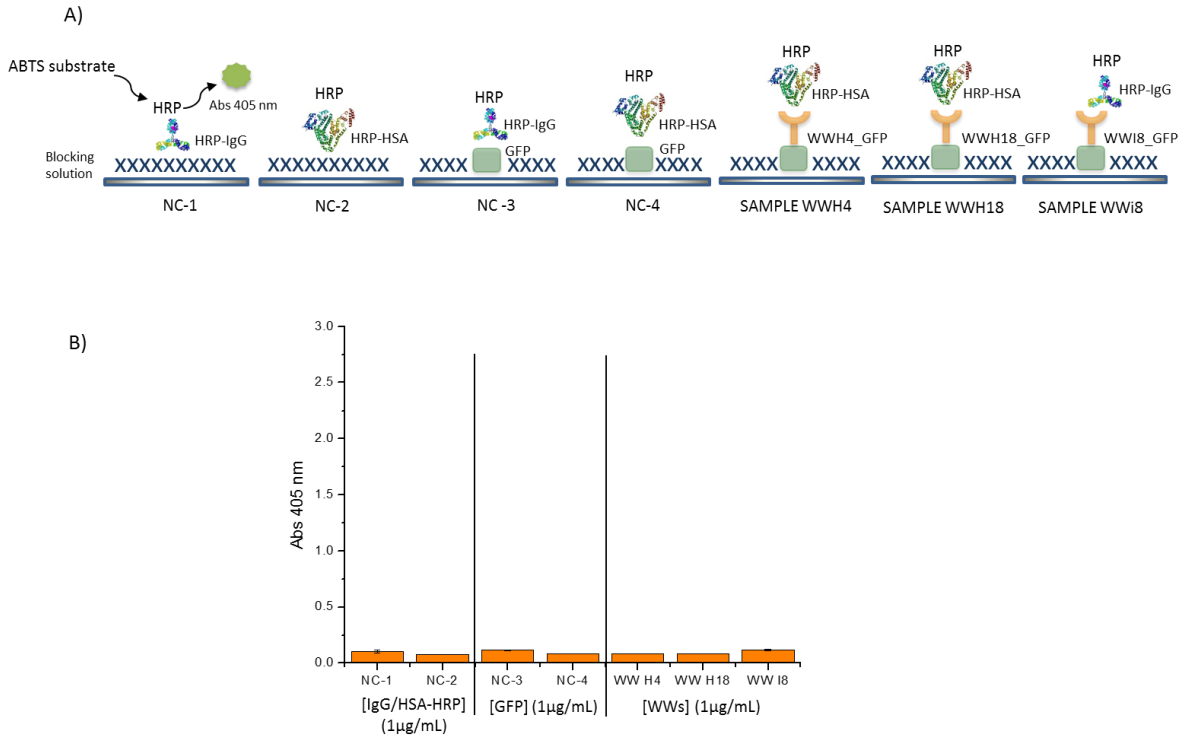


Figure 39 - Results from the direct ELISA assay with the WW prototypes. ELISA controls were used: NC-1- without target only blocking solution and HSA or IgG-HRP (NC-2) was added; Controls- GFP (1ug/well) without WW was used as negative control. (i) NC 3 GFP was immobilized in the well and was added HSA-HRP or IgG-HRP (NC-4); Samples were incubated overnight with WWs (WWH4, WWH18, WWI8) and after blocking were added HSA-HRP or IgG-HRP.

IV.5 – Conclusion

The main goal of this chapter was to produce and purify GFP and three GFP-fusion proteins WWH18, WWH4 and WWI8, and verify the specificity against the target that was selected. The fusion proteins have been previously evolved by ribosome display to bind to HSA and IgG, and selected as potential binders.

The optimal conditions of production of GFP from NZY5α competent cells was already described and for purification the ligand (C7A4) designed for this purpose was used. All the proteins have a tag with GFP so the same ligand to purify them was used.

Afterwards, with the purified proteins, a test was conducted to analyze its specificity for the target HSA or IgG.. The Enzyme-Linked Immunosorbent Assay (ELISA) was prepared in order to get the whole picture of interaction possibilities. The main goal was to observe the possible interaction between proteins and the targets that they were selected.

The first ELISA performed was a test for the process of optimization. In the end of this tests it was possible to verify that WWH18 and WWH4, lack selectivity for HSA. The protocol followed for WWI8_GFP was not sufficient to analyze the specificity because of cross-reactivity between antibodies. To overcome this problem was attempted using a direct ELISA eliminates the user antibody; another solution would be to modify the antibodies.

In this work, a direct ELISA assay was used to overcome this problem but the results were not as expected. In the future it will be important to perform a positive control to analyze the conjugation of HSA or IgG with horseradish-peroxidase (HRP).

Chapter 5.
CONCLUDING REMARKS

Human serum albumin (HSA) is the most abundant protein in the circulatory system. Because of its availability, low cost, stability, and unusual ligand binding properties, serum albumin has been one of the most extensively studied and applied proteins in biochemistry .

It is an important biomarker of many diseases, such as cancer, rheumatoid arthritis, and ischemia. Furthermore, HSA is largely used clinically to treat several diseases, like hypovolemia, shock, burns, trauma, hemorrhage, cardiopulmonary bypass, acute respiratory distress syndrome, acute liver failure, nutrition support and many others.

In 2010, 78 fractionation plants were operating in the world, 25 of which were in China, 26 in Europe and 8 in the United States of America. The world plasma fractionation capacity was about 48.4 million liters. Annually from more than 34 million liters of plasma, are produced more than 500 tons of HSA and more than 40 tons of intravenous immunoglobulins (IVIG). The average HSA consumption in the developed world is 200-400 kg per million population.

The highly abundant serum proteins (HSA and IgG), are one of the major obstacles in proteomics analysis of serum or plasma samples. Proteins present in serum have a dynamic range of concentration so it is necessary to remove proteins that mask the signal to allow detection and analysis of less abundant serum proteins. So, depletion of the most abundant proteins in human serum will significantly increase the sensitivity in analysis of the remaining proteins.

Due to high variability applications to HSA and consumption worlwide, new alternatives for the purification of HAS are needed. The aim of this work was the development of biological and synthetic affinity ligands towards HSA.

This first Chapter (Chapter 3) was a continuation of a previous work performed at the Biomolecular Engineering Lab by master student Aline Viecinski. The first part of the work was developed two affinity ligand libraries, based on Triazine and Ugi reaction. The ligands were designed to mimic the interaction between Human Serum Albumin with natural protein PAB. From the screening against HSA and IgG with these libraries resulted there lead ligands, two Triazine affinity ligand A3A2 and A6A5, and one Ugi ligand C7A3. These ligands show more specificity for HSA than for IgG. The main goal of this part was to optimize the conditions to obtain a better affinity absorbent for HSA purification.

Firstly, was synthetized the ligands and realized the screening towards HSA and IgG and was effectuated the elution step with eight different Elution Buffers, selectivity criteria were determined to select the best ligands to continue the work. Afterwards, was performed the

optimization of the binding and elution, taking to account the possible interactions the ligand can do. Hydrophobic interactions was the main interaction observe with the analyzes of ligand and HSA For the screening with plasma the affinity ligand A6A5 was used the optimized conditions **B6**: 50mM citrate buffer, 300mM NaCl pH 5 and **E10**: 25 mM sodium phosphate, 30 mM sodium caprylate pH 6 and for the ligand A3A2 was used binding buffer **B1**: 10mM Sodium phosphate, 150mM NaCl pH 7.4 at room temperature and elution buffer **E7**: 10mM PBS w/ 50 % ethylene glycol pH 7.4.Using the SDS-PAGE was confirm the presence of HSA and serum proteins in elution step was obtain 93% of purity for A6A5 and 37% for A3A2.

Afterwards, for the best affinity ligand A6A5 was determined he binding constants Ka(M^{-1}) and Qmax (mg protein bound/g support), using Langmuir model and Scatchard plot. Values are superiors obtain previous for affinity ligands immobilized on agarose

In conclusion, the synthetic ligands based on the Ugi and Triazine reactions have been successfully used to bind to HSA and the 96-well screening format proved to be a reproducible, easy to perform However, for the ligand A3A2 is important optimize the buffers or use a depletion affinity ligand for eliminate IgG. In case of ligand A6A5 once it binds 100% IgG in a future work it would be important improve the conditions to remove this protein.

The main goad of the Chapter 4 was to confirm the affinity and selectivity of 3WW sequences previously engineered and evolved in our Group to bind to HSA and IgG. Therefore, it was (i) we amplified and isolated pDNA containing the gene that expressed the protein of interest and used *E. coli* BL21 to produce crude extracts containing GFP.; (ii) produced an affinity adsorbent A4C7 ([53], for GFP purification; (iii) Purified GFP and 3GFP fusion proteins; (iv) developed ELISA assays to assess ligands binding to target.

The ELISA assay shows that the domains WWH4 and WWH18 did not bind to the HSA and for the domain WWI8 no interaction with IgG was observed. In the future it will be important to perform a control to analyze the conjugation of HSA and IgG with horseradish-peroxidase (HRP).

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Chapter 6.
ANNEX

The previous work, was performed by master student Aline Viecinski in Biomolecular Engineering Lab, and it consist on development of two affinity ligand libraries, based on Triazine and Ugi reaction. To build the combinatorial libraries, the initially study was focused on proteins that bind to the domain II of HSA, consequently was leave the domain III responsible for FcRn interaction, free to bind to the natural receptor.

The protein PAB binds in the domain II of HSA and the amino acids involved in this interaction was mimetic in order to select the amines and carboxylic acids to incorporate in libraries. To ensure greater variability of the libraries, the drugs that bind to Domain II of HSA were also included in the design

Ugi reaction is a multicomponent reaction, it is possible to incorporate three, four or even more reagents, and then have a wide variety in the final structure of the ligand. In this study, only two components, an amine (Table 18) and other carboxylic acids (Table 19) were varied binding a library with 88 different affinity ligands. The isopropyl isocyanide component was kept constant.

For the Triazine reaction, eight amines were used to construct the library leading to 64 different affinity ligands. In the triazine reaction, is indifferent use a type of amine in the first or second nucleophilic substitution, being theoretically symmetric. In the first substitution (R1), a lower temperature (30°C) is used to occur only a nucleophilic substitution between the first amine compound and the support; however, in the second substitution (R2), the temperature is increased (80°C).

All libraries were screened with pure HSA (250µg/ml) in PBS at pH 7.4.

The results obtained with the Ugi library showed that in the screening at pH 7.4 (Figure 40), the results were more promising with up to 100%, namely for A1C2, A5C2, A2C4, A3C4, A5C4 and A3C7 ligands.

In the Triazine library, when comparing with the Ugi library, more ligands with affinity to HSA were obtained. A maximum of 66% binding to HSA was observed in some ligands as A1A3, A2A3, A2A6, A3A3, A3A5, A3A6, A5A3, A5A6, A6A3, A6A2 and A6A5. In the screening at pH 7.4 (Figure 41), many ligands present 100% of HSA bound, namely ligands A1A3, A2A3,A2A5, A3A1, A3A2, A3A3, A3A4, A3A5, A3A6, A3A8, A5A3, A5A5, A5A6, A6A1, A6A2, A6A3, A6A5 and A8A3.

Table 18 - Amine compounds (A1- A8) used in the libraries of Ugi and Triazine with structure and name. The reagent who has the small rectangle indicates the amino acid which it mimics.

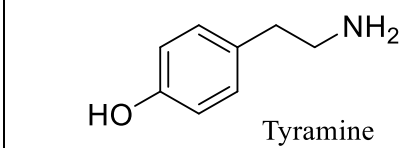
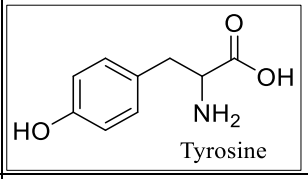
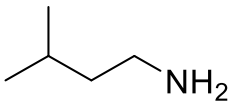
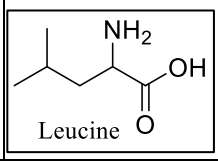
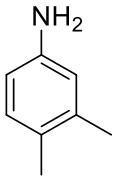
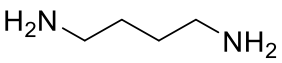
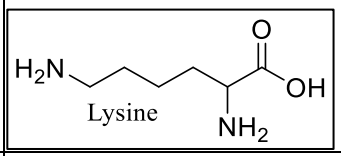
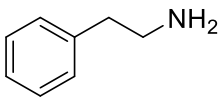
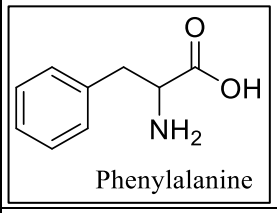
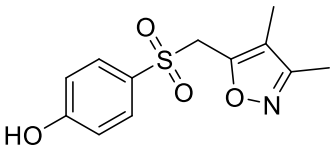
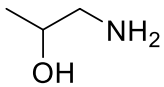
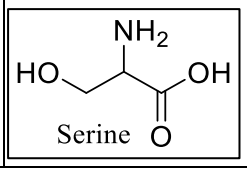
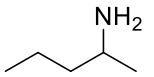
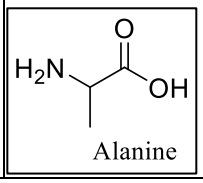
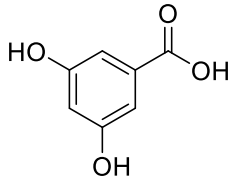
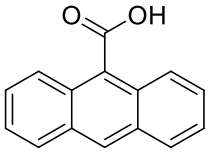
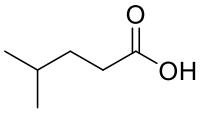
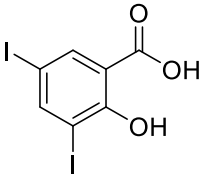
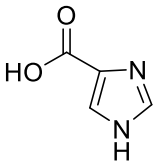
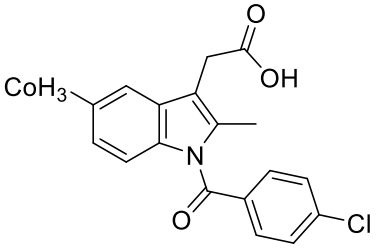
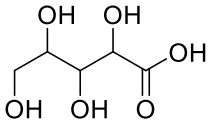
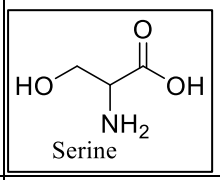
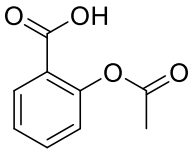
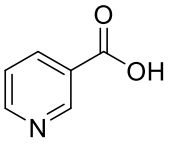
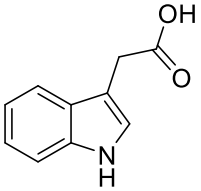
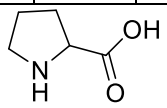
Reagent/AMINES			
A1	 Tyramine  Tyrosine	**A2**	 Isopentylamine  Leucine
A3	 3,4-Dimethylaniline	**A4**	 1,4 Diaminobutane  Lysine
A5	 Phenethylamine  Phenylalanine	**A6**	 Sulfisoxazole (Drug)
A7	 Amino-2-propanol  Serine	**A8**	 2-aminopentane  Alanine

Table 19 - Carboxylic acid compounds (C1- C11) used in the libraries of Ugi reaction with structure and name. The reagent who has the small rectangle indicates the amino acid which it mimics.

Reagent/CARBOXYLIC ACID			
C1	 3,4-dihydroxyhydrocinnamic, 98%	**C2**	 9-Anthracenecarboxylic acid
C3	 4-Methylvaleric acid	**C4**	 3,5-Diiodosalicylic acid – (Drug)
C5	 4-Imidazolecarboxylic acid	**C6**	 Indomethacin (Drug)
C7	 Arabic Acid  Serine	**C8**	 Aspirin (Drug)
C9	 Nicotinic acid	**C10**	 3-indole acetic acid
C11	 DL-Proline		

The screening of combinatorial libraries was performed with 10mM Sodium Phosphate, 150Nm NaCl pH7.4 and results are summarize in Figure 40 and 41.

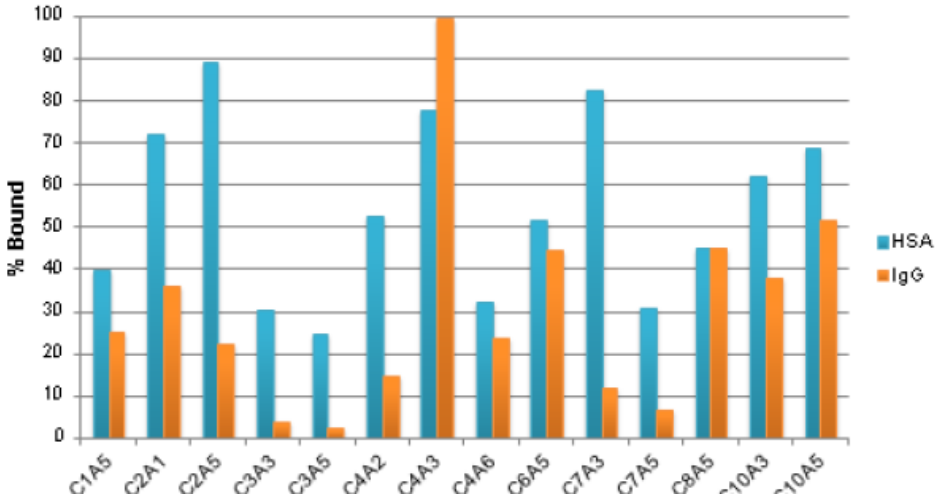


Figure 40 - Screening results of putative Ugi ligands against IgG at pH 7.4. Screening was performed by adding a loading with pure IgG and HSA (250µg/ml) in PBS (10mM Sodium Phosphate, 150mM NaCl at pH 7.4), to each well of a 96-well filtration block containing 0.25g of ligand-functionalized resin. The unbound protein from flow-through and washes was collected in 96-well transparent microplates and quantified by the QuantiProTM BCA method, measured at 560nm.

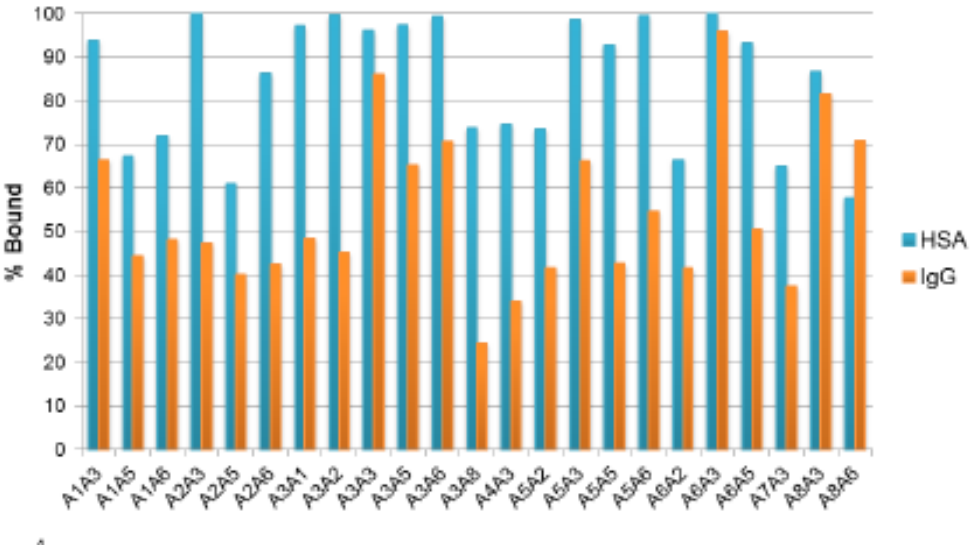


Figure 41 - Screening results of putative Triazine ligands against IgG at pH 7.4. Screening was performed by adding a loading with pure IgG and HSA (250µg/ml) in PBS (10mM Sodium Phosphate, 150mM NaCl at pH 7.4), to each well of a 96-well filtration block containing 0.25g of ligand-functionalized resin. The unbound protein from flow-through and washes was collected in 96-well transparent microplates and quantified by the QuantiProTM BCA method, measured at 560nm.

The results shows the ligands more specific for HSA than IgG, therefore was chosen two ligands from Triazine library (A3A2 and A6A5) and one for the Ugi library (C7A3).